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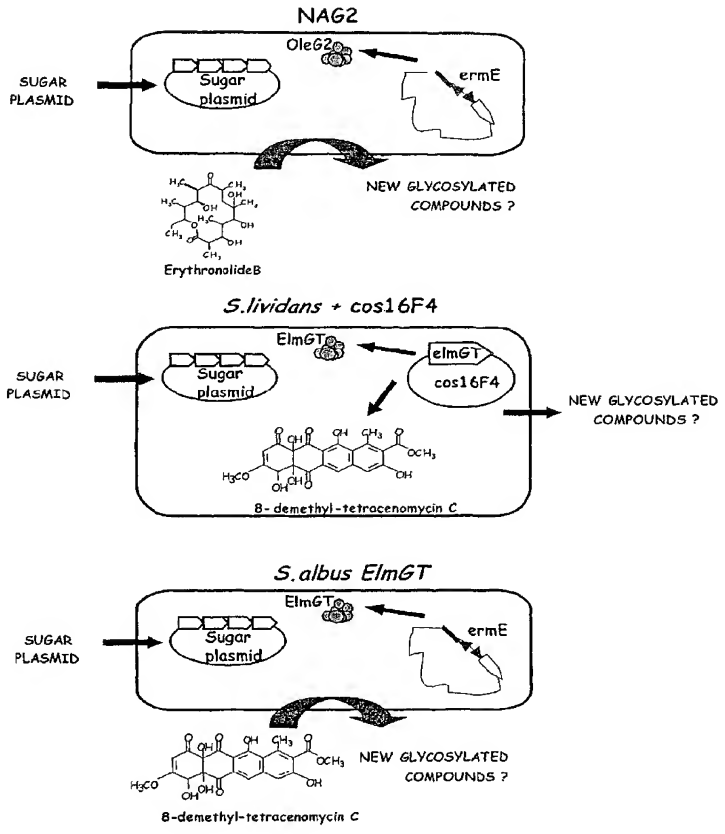
(54) Title: HYBRID GLYCOSYLATED PRODUCTS AND THEIR PRODUCTION AND USE




(57) Abstract: Hybrid glycosylated products such as polyketides and peptides are produced by transforming a host cell with (a) a gene cassette for synthesising an activated sugar and (b) nucleic acid encoding a glycosyltransferase (GT). The cell also produces or is supplied with an aglycone template. At least some of the components (sugar, aglycone, GT, sugar synthesis genes, cells) are mutually heterologous.

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# Hybrid Glycosylated Products and Their Production and Use

## Field of the Invention

The present invention relates to hybrid glycosylated products, and in particular, to natural products such as polyketides and glycopeptides, and to processes for their preparation. The invention is particularly concerned with cells containing cloned sets of biosynthetic genes for specific activated deoxysugars in a cassette format, housed for example on a plasmid. The cassette format allows convenient addition, removal or replacement of the genes in the sugar cassette so as to produce a combinatorial library of activated deoxysugars. In such cells a cloned microbial glycosyltransferase can be conveniently tested for its ability to generate specific glycosylated derivatives when supplied with polyketide, polypeptide, or polyketide-polypeptides as substrates.

## Background to the Invention

Glycosylation is important for the bioactivity of many natural products, including antibacterial compounds such as the polyketide erythromycin A and the glycopeptide vancomycin, and antitumour compounds such as the aromatic polyketide daunorubicin and the glycopeptide-polyketide bleomycin. Polyketides are a large and structurally diverse class of natural products that includes many compounds possessing antibiotic or other pharmacological properties, such as erythromycin, tetracyclines, rapamycin, avermectin, monensin, epothilones and FK506. In particular, *Streptomyces* and related genera are prodigious producers of polyketides. Polyketides are synthesised by the repeated stepwise condensation of acylthioesters in a manner analogous to that of fatty acid biosynthesis (Rawlings, 1999 and 2001a&b). The greater structural diversity found among natural polyketides arises from the selection of acyl-CoA starter units, and (generally) malonyl-CoA, methylmalonyl-CoA and ethylmalonyl-CoA as extender units; and from the differing degree of processing of the β-keto group observed after each condensation. Examples of processing steps include reduction to β-hydroxyacyl-, reduction followed by dehydration to 2-enoyl-, and complete reduction to the saturated acylthioester. The stereochemical outcome of these processing steps is also specified for each cycle of chain extension. The polyketide chains are in many cases cyclised in specific ways

and subject to further enzyme-catalysed modifications to produce the final polyketide. Naturally-occurring peptides produced by non-ribosomal peptide synthetases are likewise synthesised by repeated stepwise assembly, in this case of activated amino acids, and the chains produced are similarly subject to further modifications to produce the fully bioactive molecules (von Döhren *et al.*, 1997). Mixed polyketide-peptide compounds, hereinafter defined as incorporating both ketide and amino acid units, are also known and their bioactivity is also influenced by their pattern of glycosylation and other modification (Du *et al.*, 2001). The compounds produced by these related pathways are particularly valuable because they include large numbers of compounds of known utility, for example as anthelminthics, insecticides, immunosuppressants, antifungal or antibacterial agents.

Although large numbers of therapeutically important polyketides have been identified, there remains a need to obtain novel polyketides that have enhanced properties or that possess completely novel bioactivity. The inexorable rise in the incidence of pathogenic organisms with resistance to antibiotics such as 14-membered macrolides or glycopeptides represents a significant threat to human and animal health. Current methods of obtaining novel polyketide metabolites include large-scale screening of naturally-occurring strains of *Streptomyces* and other organisms, either for direct production of useful molecules, or for the presence of enzymatic activities that can bioconvert, to specific derivatives, a known polyketide added to the growth medium. These procedures are time-consuming and costly, and biotransformation using whole cells may in addition be limited by side-reactions or by a low concentration or activity of the intracellular enzyme responsible for the bioconversion. Given the complexity of bioactive polyketides, they are not readily amenable to total chemical synthesis in large scale. Chemical modification of existing polyketides has been widely used, but many desirable alterations are not readily achievable by these means.

Methods have been developed for the biosynthesis of altered polyketides and non-ribosomally-synthesised polypeptides by the engineering of the corresponding genes encoding the polyketide synthases and polypeptide synthetases respectively. The biosynthesis of polyketides is initiated by a group of chain-forming enzymes

known as polyketide synthases. Three classes of polyketide synthase (PKS) have been described in actinomycetes.

The first class, named Type I PKSs (Rawlings, 2001a&b) and represented by the PKSs for the macrolides erythromycin, oleandomycin, avermectin and rapamycin, consists of a different set or "extension module" of enzymes for each cycle of polyketide chain extension (Cortés *et al.*, 1990). The term "extension module" as used herein refers to the set of contiguous domains, from a β-ketoacyl-ACP synthase ("KS") domain to the next acyl carrier protein ("ACP") domain, which accomplishes one cycle of polyketide chain extension.

The modular arrangement of type-I PKSs was first confirmed by mutation of the erythromycin PKS (also known as 6-deoxyerythronolide B synthase, DEBS) through an in-frame deletion of the DNA encoding part of the ketoreductase domain in module 5 (Donadio *et al.*, 1991). This led to the production of the erythromycin analogues 5,6-dideoxy-3-β-mycarosyl-5-oxoerythronolide B and 5,6-dideoxy-5-oxoerythronolide B due to the inability of the mutated KR domain of module 5 to reduce the β-keto group introduced at this stage of the processive biosynthesis. Likewise, alteration of active site residues in the enoylreductase domain of module 4 in DEBS, by genetic engineering of the corresponding PKS-encoding DNA and its introduction into *Saccharopolyspora erythraea*, led to the production of 6,7-anhydroerythromycin C (Donadio *et al.*, 1993). WO 93/13663 describes additional types of genetic manipulation of the DEBS genes that are capable of producing altered polyketides. However, many such attempts are reported to have been unproductive (Hutchinson & Fujii, 1995).

WO 98/01546 describes the engineering of hybrid Type I PKS genes which utilise portions of PKS genes derived from more than one natural PKS, particularly derived from different organisms, and the use of such recombinant genes for the production of altered polyketide metabolites.

The second class of PKS, named Type II PKSs, is represented by the synthases for many aromatic compounds produced by bacteria. Type II PKSs contain only a single set of enzymatic activities for chain extension and these are re-used in successive cycles (Bibb *et* al., 1989; Sherman *et* al., 1989; Fernandez-Moreno *et al.*, 1992). The extender units for the Type II PKSs are usually malonyl-CoA units, and the presence of specific cyclases dictates the preferred pathway for cyclisation of the completed chain into an aromatic product (Hutchinson & Fujii, 1995). Hybrid polyketides have been obtained by the introduction of cloned Type II PKS gene-containing DNA into another strain containing a different Type II PKS gene cluster, for example, by introduction of DNA derived from the gene cluster for actinorhodin, a blue-pigmented polyketide from *Streptomyces coelicolor*, into an anthraquinone polyketide-producing strain of *Streptomyces galileus* (Bartel *et al.*, 1990).

The minimal number of domains required for polyketide chain extension on a Type II PKS when expressed in a *Streptomyces coelicolor* host cell (the "minimal PKS") has been defined (for example in WO 95/08548) as containing the following three polypeptides which are products of the *act I* genes: first a KS; secondly a polypeptide termed the CLF with end-to-end amino acid sequence similarity to the KS but in which the essential active site residue of the KS, namely a cysteine residue, is substituted either by a glutamine residue, or in the case of the PKS for a spore pigment such as the *whiE* gene product (Chater & Davis, 1990) by a glutamic acid residue; and finally an ACP. The CLF has been stated (for example in WO 95/08548) to be a factor that determines the chain length of the polyketide chain that is produced by the minimal PKS. However, it has been found (Shen *et al.*, 1995) that when the CLF for the octaketide actinorhodin is used to replace the CLF for the decaketide tetracenomycin in host cells of *Streptomyces glaucescens*, the polyketide product is not found to be altered from a decaketide to an octaketide. An alternative nomenclature has been proposed in which KS is designated $KS_\alpha$ and CLF is designated $KS_\beta$, to reflect this lack of confidence in the correct assignment of the function of CLF (Meurer *et al.*, 1997). WO 00/00618 has recently shown that CLF and its counterpart in Type I PKS multienzymes, the so-called KSQ domain, are involved in *initiation* of polyketide chain synthesis. WO 95/08548 for example

describes the replacement of actinorhodin PKS genes by heterologous DNA from other Type II PKS gene clusters, to obtain hybrid polyketides.

The third class of PKS, named Type III PKSs, is represented by the synthases for certain other aromatic compounds in bacteria, such as flaveolin from *Streptomyces griseus* (Funa *et al.*, 1999). Type III PKSs contain only a single active site where chain extension occurs. The "extender" units for the Type III PKSs are usually acetate units, and the shape and volume of the active site apparently dictate the preferred pathway for cyclisation of the completed chain into an aromatic product (Jez *et al.*, 2000). The product of a Type III PKS has been successfully altered by specific mutagenesis of portions of the enzyme structure, based on a knowledge of the X-ray crystal structure of typical chalcone synthases and stilbene synthases (Ferrer *et al.*, 1999). The aromatic products of Type III PKSs are often further processed by oxidation, and deoxsugar attachement may also occur (Cortes *et al.*, 2002).

This ability to engineer PKS genes of all Type I, Type II and Type III origin raises the possibility of the combinatorial biosynthesis of polyketides to produce diverse libraries of novel natural products which may be screened for desirable bioactivities. However, the aglycones produced by recombinant PKS genes may or may not be partially processed by glycosyltransferases and/or other modifying enzymes into analogues of the mature polyketides. There is therefore an additional need to provide processes for efficient conversion of such novel aglycones into specific glycosylated products. Further, the invention of efficient processes for glycosylation would provide a new means to increase very significantly the diversity of combinatorial polyketide libraries, by utilisation of recombinant cells containing alternative cloned glycosyltransferases and alternative complements of activated sugars (Salas and Méndez, 2001; Rohr *et al.*, 2002).

The well-known influence of glycosylation on biological activity has encouraged intensive research into the genes and enzymes governing the synthesis and attachment of specific sugar units to polyketide and polypeptide metabolites (for a review see Trefzer *et al.*, 1999). Surveys of such metabolites have revealed a high diversity in

the type of glycosyl substitution that is found, including a very large number of different deoxyhexoses and deoxyaminohexoses (see for a review Liu & Thorson, 1994). The sequencing of biosynthetic gene clusters for numerous glycosylated polyketides, polypeptides and mixed polyketide/peptide systems has revealed the presence of such sugar biosynthetic genes. In addition, genes encoding the glycosyltransferases that transfer the glycosyl group from an activated form of the sugar, eg dTDP- or dUDP- forms, to the aglycone acceptor have also been identified. For example, the *eryB* genes and the *eryC* genes of the erythromycin biosynthetic gene cluster in *Saccharopolyspora erythraea* have been identified as involved in the biosynthesis and attachment to the aglycone precursor of erythromycin A of L-mycarose and D-desosamine, respectively (Dhillon *et al.*, 1989; Haydock *et al.*, 1991; Salah-Bey *et al.*, 1998; Gaisser *et al.*, 1998; Gaisser *et al.*, 1997; Summers *et al.*, 1997). Both WO 97/23630 and WO 99/05283 describe the preparation of an altered erythromycin by deletion of a specific sugar biosynthetic gene, so that an altered sugar becomes attached to the aglycone. Thus WO 99/05283 describes low but detectable levels of erythromycins in which, for example, desosamine is replaced by mycaminose (*eryCIV* knockout), or desmethylmycarosyl erythromycins (*eryBIII* knockout) are produced. Meanwhile methymycin analogues have been produced in which desosamine has been replaced by D-quinuvose (Borisova *et al.*, 1999) or which display altered patterns of amino group substitution through the incorporation of the *calH* gene of the calicheamycin gene cluster from *Micromonospora echinospora* into the methymycin producing strain (Zhao *et al.*, 1999). Similarly, hybrid glycopeptides have been produced by using cloned glycosyltransferases from the vancomycin-producer *Amycolatopsis orientalis* to add D-xylose or D-glucose to aglycones of closely-related glycopeptides according to US 5,871,983 (see also Solenberg *et al.*, 1997). Hybrid aromatic polyketides have also been produced by interspecies complementation, by utilising a homologue of a biosynthetic gene that performs an analogous chemical reaction, but with a different stereospecificity. Thus, instead of the natural daunosamine, 4'epi-daunosamine is produced in recombinant *Streptomyces peucetius* and attached by the daunosamine glycosyltransferase to the natural aglycone, to yield the antitumour derivative epirubicin in place of doxorubicin (Madduri *et al.*, 1998).

In all these cases, the broad specificity of the glycosyltransferase allowed the substitution of an alternative activated sugar, but the aglycone and glycosyltransferase were not heterologous to each other. It has been found that when the oleandrose glycosyltransferase OleG2, from the oleandomycin biosynthetic gene cluster of *Streptomyces antibioticus*, is cloned into the erythromycin-producing strain *S. erythraea*, in addition to other products, a novel erythromycin in which the L-cladinose moiety at C-3 is replaced by L-rhamnose was obtained (Doumith *et al.*, 1999). It was assumed that the activated L-rhamnose is produced by the host cells, and is recruited by OleG2 in competition with the activated L-mycarose known to be present.

The sugar D-desosamine is found attached to many macrolides (Trefzer *et al.*, 1999) and it has been found that biosynthetic genes for the biosynthesis of the activated form of this sugar are accordingly found in each of the gene clusters for such macrolides. Similarly the sugar L-oleandrose is found for example in oleandomycin produced by *S. antibioticus*, while two such oleandrosyl moieties are present in avermectins produced by *Streptomyces avermitilis*. Comparison of gene sequences and specific gene disruptions has led to the recognition that in *S. avermitilis* eight genes from the avermectin cluster (*avrBCDEFGHI*) are involved in the biosynthesis and attachment of L-oleandrose (for a review see Wohlert *et al.*, 2001). The AvrD and AvrC enzymes control production of dTDP-4-keto-6-deoxyglucose; C-2 deoxygenation occurs next catalyzed by AvrG and AvrI; it is proposed that this is followed by 5-epimerisation by AvrF, O-methylation at C-3 catalysed by AvrH, and finally 4-ketoreduction by AvrE to produce dTDP-L-oleandrose. The same order of events has been proposed in the oleandomycin producer (Aguirrezbalaga *et al.*, 2000) but more recently it has become clear that methylation in this case does not occur until the neutral sugar is attached to the macrolide aglycone (Rodriguez *et al.*, 2001) so that the sugar actually transferred by the glycosyltransferase in the normal pathway is L-olivose.

As a result of such analyses, the use of plasmid systems to express deoxysugar biosynthetic pathways in a heterologous host is well known in the art. Using genes derived from the oleandomycin biosynthetic gene cluster in *S. antibioticus*, a plasmid (pOLV) containing a set of genes proposed to be required for the biosynthesis of dTDP-L-olivose and a second plasmid (pOLE) containing a set of genes proposed to be required for the biosynthesis of dTDP-L-oleandrose were constructed and introduced into a strain of *Streptomyces albus* which is not known to synthesise either these sugars or any macrolide polyketide, and which contains the *oleG2* gene integrated into the genome. When supplied with the heterologous macrolide aglycone erythronolide B, 3-O-L-olivosyl erythronolide B was produced from the strain housing pOLV, and 3-O-L-oleandrosyl erythronolide B was produced from the strain housing pOLE. These results indicate that the gene sets carried by the respective plasmids are necessary and sufficient to produce the appropriate activated sugar substrate for the glycosyltransferase in a heterologous host strain (Aguirrezabalaga *et al.*, 2000). In these plasmids the genes were largely in their natural gene order and expressed from their native promoters.

It has been shown more recently that the equivalent of these same eight genes from the avermectin biosynthetic pathway of *S. avermitilis* (*avrBCDEFGHI*) can be similarly expressed in a heterologous host under the control of their own promoters (Wohlert *et al.*, 2001). They can also be expressed from a heterologous promoter, specifically that for the erythromycin resistance gene *ermE** from *S. erythraea*. It was additionally shown that these oleandrose biosynthetic genes could be grouped into two distinct artificial operons, or expression cassettes, on a plasmid under the control of the overlapping divergent heterologous *actI/actIII* promoters from *Streptomyces coelicolor*, and that this led to the production of dTDP-oleandrose in *Streptomyces lividans*. Other versions of the expression plasmid were synthesised *de novo* in which individual oleandrose biosynthetic genes were eliminated from one cassette by in frame deletion; or omitted from the second cassette during construction. Avermectin aglycone was converted by *S. lividans* strains containing plasmids bearing such modified expression cassettes into glycosylated avermectin analogues. Thus, elimination of the gene *avrF*, the presumed 5-epimerase, led to the attachment of a D-

sugar, D-olivose, to the aglycone. In other examples, mixtures of products were formed: for example, deletion of the presumed methyltransferase gene (*avrH*) led to glycosides with L-olivose or alternatively L-digitoxose (differing from L-olivose in having an inverted configuration at C-3) attached in place of oleandrose. The methylation was restored at low levels by complementing the latter strain with the 3-C-methyltransferase gene *eryBIII* to give so far uncharacterised avermectins. EryBIII operates within the biosynthetic pathway for the production of a closely related neutral deoxyhexose, mycarose, in the erythromycin producing strain of *S. erythraea*; *eryBIII* was introduced into the *S. lividans* host on a separate plasmid.

Despite these insights there remains considerable uncertainty over the identity and role of individual genes in the biosynthesis of many activated deoxy- and dideoxyhexoses. Further, there is considerable labour involved in the construction of the plasmid systems and great uncertainty over whether a particular combination of genes when cloned in a particular order will be properly expressed and will function as in their native context. Additionally, there is an urgent need for new methodologies for more rapid and convenient cassette-based expression of such sugar pathway genes, in particular in a manner that allows the rapid and flexible substitution, removal, addition, or rearrangement of the genes in the cassette in order to test and optimise the combinations of genes being used. In many of the experiments reported in the art, the degree of bioconversion was extremely limited and would not form the basis of any useful process for preparation of polyketide drugs. The reasons for this are unknown and may be complex, but it is obvious that one root of this problem could lie in the suboptimal performance of the various arbitrarily-constructed cassettes.

## Summary of the Invention

The present invention shows that plasmid-based gene cassettes can be constructed which direct the synthesis of different dTDP sugars in a rationally designed and easy manner, with the sugar genes in each case flanked by unique restriction sites that facilitate gene exchanges. The design also allows the flexible and easy sequential linking of individual genes to build up the original gene cassette. The plasmid also has a unique *Xba*I site that can be used to co-clone additional genes with functions not

present in the original plasmid. If such additional genes are cloned into the cassette plasmid using either *Spe*I, *Avr*II or *Nhe*I for the 5′-end and *Xba*I for the 3′-end of the gene then a unique *Xba*I restriction site is preserved for further use.

In the creation of the plasmid-based gene cassettes that are the subject of the invention, the genes may be cloned together from their natural context, as for example the genes for the initial three steps common in L-deoxysugar biosynthesis (dTDP-glucose synthase, *oleS*; 4,6-glucose dehydratase, *oleE*; 3,5-epimerase, *oleL*) (Figure 2) which were cloned on a single fragment in the example of cassette plasmid pLN2 described below. Alternatively and preferably, they may be individually generated by PCR and flanked by unique restriction sites introduced by the PCR process. Each amplified gene contains its own ribosomal binding site (Table 2). Two of the unique restriction sites (*Hind*III and *Xba*I) are used to facilitate subcloning of each amplicon in *E. coli* vectors (eg. pUC18) in order to generate a sugar gene library. The other two restriction sites are used to facilitate the sequential addition of sugar genes in order to generate the initial plasmid construct. Also, these restriction sites allow exchange of genes with similar functions in order either to confirm function, or to assay the substrate flexibility of the different enzymes. These restriction sites were selected since they cut very rarely in DNA of high G+ C content typical of actinomycetes. Thus, for example, the 4-ketoreductase gene *oleU* can be replaced by one of a number of other natural 4-ketoreductase genes, which may subsequently lead to the formation of different product activated sugars (Figure 8). In one of the examples described here the use of the gene *eryBIV* leads to the production of the unusual sugar L-digitoxose (Figure 9), with its subsequent attachment to an aglycone, in good yield. If the basic cassette, for example the plasmid pLN2 (Figures 3A, 4, and 6) whose construction is given below, directs the biosynthesis of 2,6-dideoxysugars, then to generate cassette plasmids for 6-deoxysugars, the two genes involved in the 2-deoxygenation process in sugar biosynthesis (*oleV* and *oleW*) can be easily removed. Specifically, for example using the two unique restriction sites (*Avr*II and *Spe*I) flanking this pair of genes (Figures 6 & 11), a plasmid pLN2Δ can be generated which is competent for the biosynthesis of L-rhamnose.

Using such plasmid-based gene cassettes, glycosyltransferase enzymes can be rapidly screened for their ability to attach a range of activated sugars to a range of exogenously supplied, or endogenously generated, aglycone templates. Patent application WO 01/79520 for example demonstrates that such glycosyltransferases frequently show surprising flexibility towards both aglycone and sugar substrates, and that this process allows the production of novel glycosylated polyketides in good yield. This overcomes the problem not only of supplying novel sugar attachments to individual polyketides, including polyketides altered by genetic engineering, but also of increasing the diversity of polyketide libraries by combinatorial attachment of sugars. It is particularly surprising that new glycosylated products can be produced in systems in which one or more of the components are heterologous to each other, the components being selected from the aglycone template, the sugar moiety or moieties, the glycosyltransferase, the host cell and/or genes encoding enzymes capable of modifying the sugar moiety, either before or after attachment to the aglycone template. In preferred embodiments, two, three, four, or all of the components are heterologous to each other.

It will readily occur to those skilled in the art that the methods described here can be readily extended to embrace alternative sugar genes from other natural sugar biosynthetic pathways. Examples of such pathways, for which detailed information is already available, include the sugars desosamine, mycarose, mycaminose, rhodinose, oliose, olivose and oleandrose. There is also the opportunity to select the nature of the activated sugar to be either in the L- or in the D- form. The cassette arrangement for several of these further sugars is shown in Figures 13 and 14.

It will be additionally readily apparent that even in those cases where the initial screening for the presence of the predicted activated sugar product did not show its transfer to the provided aglycone, this may simply reflect the inability of the glycosyltransferase to catalyse that particular transformation, rather than the non-functionality of the sugar cassette itself. Therefore, as the lexicon of glycosyltransferases is expanded, and as knowledge increases about their range of substrate specificity, so the useful range of plasmid-based gene cassettes encoding the

biosynthesis of deoxyhexose (sugar) templates constructed as described here will correspondingly increase.

Accordingly, in a first aspect, the present invention provides a process for producing a hybrid glycosylated product by transferring one or more sugar moieties to an aglycone template, the process comprising:

transformation of microorganism host cells with nucleic acid encoding a plasmid-based gene cassette which contains the genes sufficient to direct the synthesis of a specific activated sugar in those host cells; and also with nucleic acid encoding a glycosyltransferase (GT); and,

providing an aglycone template to the transformed microorganism so that the GT transfers one or more sugar moieties to the aglycone template in order to produce a hybrid glycosylated product;

wherein one or more of the sugar moieties, the aglycone template, the glycosyltransferase, the genes encoding enzymes capable of modifying sugar moieties or the host cells are heterologous to the other components of the invention.

In a further aspect, the present invention provides host cells transformed with nucleic acid encoding a gene expression cassette which contains the genes sufficient to direct the synthesis of a specific activated sugar in those host cells, optionally operably linked under the same promoter; and also transformed with a glycosyltransferase (GT), wherein the GT is heterologous to the host cells and transfers one or more sugar moieties to an aglycone template within the cells to produce a hybrid glycosylated product. Examples of specific activated deoxysugars include, but are limited to the following: L-NDP-olivose, L-NDP-oleandrose, L-NDP-oliose, L-NDP-mycarose, L-NDP-cladinose, L-NDP-digitoxose, L-NDP-3-O-methyldigitoxose, L-NDP-rhamnose, L-NDP-2-O-methylrhamnose, L-NDP-3-O-methylrhamnose, L-NDP-4-O-methylrhamnose, L-NDP-2,3-di-O-methylrhamnose, L-NDP-2,3,4-tri-O-methylrhamnose, L-NDP-rhodinose

In a further aspect, the present invention provides a process for producing a hybrid glycosylated product, the process comprising culturing the host cells defined above and isolating the product thus produced. In embodiments in which the aglycone template is supplied to the host cells, rather than being produced by the host cell, the process may comprise the additional step of supplying the aglycone template to the cells.

In further aspects, the present invention provides hybrid glycosylation products as obtainable by any of the processes disclosed herein.

Embodiments of the present invention will now be described by way of example, and not limitation, with reference to the accompanying figures.

**Brief Description of the Figures**

Figure 1: Structure of oleandomycin.

Figure 2: Organisation of the genes required for dTDP-L-oleandrose biosynthesis from the gene cluster of *Streptomyces antibioticus* governing the biosynthesis of oleandomycin.

Figure 3: Scheme for the PCR amplification and cloning of individual genes encoding enzymes that catalyse steps in deoxysugar biosynthesis to create sugar gene libraries, and their incorporation stepwise into an expression cassette plasmid.

Figure 4: Detailed scheme for construction of plasmid pLN1.

Figure 5: Scheme showing the TLC based screening of plasmid pLN1 housed in *Streptomyces albus* GB16 for its ability to produce activated L-olivose which is then attached to erythronolide B in the presence of the glycosyltransferase OleG2. Because the methyltransferase OleY is also present the attached sugar is subsequently methylated to provide oleandrosyl-erythronolide B.

Figure 6: Scheme showing the derivation of expression cassette plasmid pLN2 in which the *Xba*I site is now unique; and the scheme for extrusion of *oleV* and *oleW* genes to create expression cassette plasmid pLN2ΔVW which provides for synthesis of dTDP-L-rhamnose.

Figure 7: Schemes showing the methods used to test for the intracellular production of activated sugars, through the catalysis of their attachment to suitable aglycones in the presence of a suitable glycosyltransferase. The glycosyltransferase ElmGT for example is known to transfer L-rhamnose, L-olivose, L-rhodinose, D-olivose and D-mycarose to the aglycone 8-demethyltetracenomycin C.

Figure 8: Scheme showing the exchange of a heterologous 4-ketoreductase gene for the oleU 4-ketoreductase gene in plasmid pLN2.

Figure 9: Scheme showing the natural substrate for 4-ketoreductase EryBIV and alternative substrates for this enzyme.

Figure 10: Products of the fermentation of strain LI16 containing plasmid pLN2EryBIV.

Figure 10A: HPLC analysis of the production of glycosylated products in the strain NAG2 (containing the glycosyltransferase OleG2, as well as the plasmid pLN2EryBIV, and supplied with Erythronolide B.

Figure 11: Scheme showing the deletion of genes oleV and oleW from plasmid pLN2.

Figure 12: Products containing L-rhamnose from the fermentation of strains containing plasmid pLN2ΔVW.

Figure 12A: production of glycosylated products in the strain *S. albus* NAG2 (containing the glycosyltransferase OleG2, as well as the plasmid pLN2ΔVW, and supplied with erythronolide B.

Figure 13: Scheme showing the arrangement of genes in the expression cassette plasmid pDES.

Figure 14: Construction of expression cassette plasmids for the production of dTDP-L-olivose and dTDP-L-mycarose.

Figure 15: Shows the arrangement of genes in the expression plasmid PKS.

Figures 16 and 17 are diagrams for use in describing and explaining the construction of the plasmids described in this patent.

Table 1: Genes used in the construction of gene cassette plasmids.

Table 2: Synthetic oligonucleotides used in the PCR amplification of individual deoxysugar pathway genes for construction of pathway expression gene cassettes. Each oligonucleotide contains both general and specific restriction sites, and the forward primers contain the requisite ribosomal binding site motif and start codon.

Table 3: Plasmid constructs directing the biosynthesis of different sugars and glycosyltransferase (GTF) systems used to show the presence of the sugar.

**Methods and Materials**

*Microorganisms, culture conditions and vectors*

*Streptomyces antibioticus* ATCC11891 (oleandomycin producer), *Streptomyces fradiae* ATCC19609 (tylosin producer), *Streptomyces peucetius* ATCC29050 (daunorubicin producer), *Streptomyces nogalater* NRRL3035 (nogalamycin producer), and *Saccharopolyspora erythraea* NRRL2338 (erythromycin producer) were used as source of DNA. *Streptomyces albus* GB16 (Blanco *et al.*, 2001) was used as host for gene expression and for biotransformation experiments. *Streptomyces argillaceus* 16F4 (Blanco *et al.*, 2001) was used for obtaining 8-demethyl-tetracenomycin C (8DMTC). Bacterial growth was carried out on trypticase soya broth (TSB; Oxoid) or R5A medium (Fernández *et al.*, 1998). To obtain spores growth was carried out for 7 days at 30°C on agar plates containing A-medium (Fernández *et al.*, 1998). *Escherichia coli* XL1-Blue (Bullock *et al.*, 1987) was used as a host for subcloning and was grown at 37°C in TSB medium. pLITMUS29 (Biolabs) and pUC18 were used as vectors for subcloning experiments and DNA sequencing. pWHM3 (Vara *et al.*, 1989) and pEM4 (Quirós *et al.*, 1998) were used for expression in *Streptomyces*. Where antibiotic selection was required thiostrepton (25 μg/ml), apramycin (25 μg/ml) or ampicillin (100 μg/ml) were used.

*DNA manipulation and sequencing*

Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, ligations and other DNA manipulations were performed according to standard procedures for *E. coli* (Sambrook *et al.*, 1989) and for *Streptomyces* (Kieser *et al.*, 2000). DNA sequencing was performed using the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) and the Cy5 AutoCycle Sequencing Kit (Pharmacia Biotech). Both DNA strands were sequenced with primers supplied in the kits or with internal oligoprimers (17-mer) using an ALF-express automatic DNA sequencer (Pharmacia). Computer-assisted data base searching and sequence analyses were carried out using the University of Wisconsin Genetics Computer Group programs package (UWGCG; Devereux *et al.*, 1984) and the BLASTP program (Altschul *et al.*, 1990).

*PCR amplification of the genes*

Individual genes were amplified by PCR using the oligonucleotide primers listed in Table 2. These primers were designed to create *Hind*III and *Xba*I sites at the 5'-end and 3'-end respectively of all genes in order to facilitate subcloning. Moreover, two other restriction sites were included in each pair of oligoprimers which were specific for each gene, to facilitate the exchange of specific genes. PCR reaction conditions were as follows: 100 ng of template DNA were mixed with 30 pmol of each primer and 2 units of Vent DNA Polymerase (New England Biolabs) in a total reaction volume of 50 µl containing 2mM of each dNTP, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton X-100 and 10% DMSO (Merck). This reaction mix was overlaid with 50 µl of mineral oil (Sigma) and the polymerization reactions were performed in a thermocycler (MinyCycler, MJ Research). The PCR products were purified and subcloned into pUC18.

*Construction of cassette plasmids*

Two gene cassette plasmids were initially constructed:

Plasmid pLN1: for constructing this plasmid, the oleandomycin sugar genes, *oleV*, *oleW*, *oleU* and *oleY*, were independently amplified using the oligoprimers described in Table 1 and the conditions above. Subsequently, all the genes were sequentially cloned into pUC18 to generate pUC18VWUY. To achieve this, each gene was subcloned using the 5' specific restriction site and the *Xba*I site (located at 3'-end of each gene) into intermediate plasmid constructs which were digested with the same restriction enzymes. Then, the *Avr*II-*Xba*I fragment containing the four genes was cloned downstream of the erythromycin resistance promoter of pEM4, generating pLN. Finally, a *Spe*I-*Xba*I fragment (using these sites from the polylinker) containing *oleL*, *oleS* and *oleE* was subcloned from pLR234Δ7 (Rodríguez *et al.*, 2000) into the *Xba*I site of pLN, generating pLN1.

Plasmid pLN2. First, the *oleV* PCR fragment was subcloned as an *Avr*II-*Xba*I fragment into the same sites of pLITMUS29 and rescued as a *Spe*I (using this site from the polylinker)-*Xba*I fragment for subcloning into the *Xba*I site of pEM4, downstream of the erythromycin resistance promoter, generating pEM4V. Then, *oleV* in pLN1 was

exchanged by *oleV* from pEM4V generating pLN1b, by digesting both constructs with *Hind*III and *Hpa*I. Finally the *Hind*III-*Xba*I fragment of pLN1b that contains all of the oleandrose biosynthetic genes from *S. antibioticus* under the control of the erythromycin resistance promoter, was subcloned into the same sites of pWHM3, generating plasmid pLN2.

Various derivatives of plasmid pLN2 were made as follows:

Plasmid pLN2ΔVW: Plasmid pLN2 was digested with *Avr*II and *Spe*I and the larger of the two DNA fragments produced was purified by gel electrophoresis and then ligated to itself (as *Avr*II and *Spe*I have compatible ends) to obtain the desired plasmid pLN2ΔVW.

Plasmids pLN2 EryBIV, pLNSnogC, pLNDnmV, pLNTylD: Plasmid pLN2 was digested with *Spe*I and *Nhe*I and the larger of the two DNA fragments produced was isolated. The *eryBIV* gene was amplified by PCR using the oligonucleotide primers 5'-B4U and 3'-B4L listed in Table 2, and cloned into pUC18. The *eryBIV* gene insert was excised from this clone and ligated with the *Spe*I-*Nhe*I digested pLN2 backbone to create plasmid pLN2EryBIV. Similarly, the *snogC* gene from *S. nogalater*, the *dnmV* gene *of S. peucetius*, and the *tylD* gene of *S. fradiae* were each amplified using the appropriate forward and reverse oligonucleotide primers listed in Table 2, and cloned into pUC18. The respective gene inserts were excised from these clones and ligated into the *Spe*I-*Nhe*I digested backbone of pLN2 to create plasmids pLN2SnogC, pLN2DnmV and pLN2TylD respectively.

*Transformation of biotransformation host cells*

The plasmid based gene cassettes and glycosyltransferases were introduced into the two biotransformation host organisms *S. lividans* TK21 and *S. albus* GB16 by protoplast transformation according to the standard procedures (Kieser *et al.*, 2000).

*Biotransformation and chromatographic techniques*

Spores of the appropriate *S. albus* GGB16 or *S. lividans* TK24 recombinant strains carrying the relevant GT and plasmid-based gene cassette for deoxysugar biosynthesis were grown in the presence of 8-demethyl-tetracenomycin C (Blanco *et al.*, 2001) or erythronolide B (Aguirrezabalaga *et al.*, 2000) according to conditions previously described. TLC, HPLC and LCMS analyses were performed as previously described (Aguirrezabalaga *et al.*, 2000; Fernández-Lozano *et al.*, 2000; Blanco *et al.*, 2001).

*Specific examples of products identified from engineered biotransformation strains*

Example 1: Glycosylation reporter systems

The glycosyltransferase, ElmGT, of the elloramycin pathway, has broad substrate specificity towards several L-6-deoxysugars (L-olivose, L-rhamnose, L-rhodinose) and D-6-deoxysugars (D-olivose, D-mycorose). ElmGT is capable of transferring such sugars onto the aglycone 8-demethyl-tetracenomycin C (8DMTC) (Fig. 7). The glycosyltransferase, OleG2, of the oleandomycin pathway, has broad substrate specificity towards several 6-deoxysugars. OleG2 is capable of transferring such sugars onto the aglycone erythronolide B (EB) (Fig. 7). The glycosyltransferase, EryBV, of the erythromycin pathway, has broad substrate specificity towards several 6-deoxysugars. EryBV is capable of transferring such sugars onto the aglycone EB (Fig. 7).

a. *Provision of endogenous 8DMTC in* S. albus.

*S. albus* 16F4, a recombinant strain harbouring cosmid 16F4 that directs endogenous biosynthesis of 8DMTC and contains *elmGT*, provides a reporter strain that when transformed with pLN2 derivatives allows the detection of plasmid-directed synthesis of NDP-6-deoxysugars by their transfer to 8DMTC and the subsequent formation of the corresponding glycosylated compound (Fig.7).

b. *Provision of endogenous 8DMTC in* S. lividans.

*S. lividans* LI16, a recombinant derivative strain of *S. lividans* TK21 harbouring cosmid 16F4 that directs endogenous biosynthesis of 8DMTC and contains *elmGT*, provides a reporter strain that when transformed with pLN2 derivatives allows the detection of plasmid-directed synthesis of NDP-6-deoxysugars by their transfer to 8DMTC and the subsequent formation of the corresponding glycosylated compound (Fig.7).

*c.* S. albus *GB16, a biotransformation strain for 8DMTC*

*S. albus* GB16, a recombinant strain with *elmGT* integrated into the chromosome and expressed under the control of *ermE** (the erythromycin resistance promoter of *S. erythraea*), provides a reporter strain that when transformed with pLN2 derivatives and fed the aglycone 8DMTC allows the detection of plasmid-directed synthesis of NDP-6-deoxysugars by their transfer to 8DMTC and the subsequent formation of the corresponding glycosylated compound (Fig.7).

*d.* S. lividans *NAG2, a biotransformation strain for 8DMTC or erythronolide B.*

*S. lividans* NAG2, a recombinant strain with *oleG2* integrated into the chromosome and expressed under the control of *ermE** (the erythromycin resistance promoter of *S. erythraea*), provides a reporter strain that when transformed with pLN2 derivatives and fed the aglycone 8DMTC or erythronolide B (EB) allows the detection of plasmid-directed synthesis of NDP-6-deoxysugars by their transfer to 8DMTC or EB and the subsequent formation of the corresponding glycosylated compound (Fig.7).

*e.* S. lividans *NAB5, a biotransformation strain for 8DMTC or erythronolide B.*

*S. lividans* NAB5, a recombinant strain with *eryBV* integrated into the chromosome and expressed under the control of *ermE** (the erythromycin resistance promoter of *S. erythraea*), provides a reporter strain that when transformed with pLN2 derivatives and fed the aglycone 8DMTC or erythronolide B (EB) allows the detection of plasmid-directed synthesis of NDP-6-deoxysugars by their transfer to 8DMTC or EB and the subsequent formation of the corresponding glycosylated compound.

Example 2: Functionality of pLN2

*S. albus* 16F4, *S. albus* GB16 and *S. lividans* NAG2 were transformed with pLN2 encoding the biosynthetic pathway for the production of L-olivose (see Materials and Methods above). *S. albus* 16F4 + pLN2 was found to produce a compound consistent with L-olivosyl-tetracenomycin C (LOLV-TCMC) in terms of HPLC mobility and absorption spectrum when compared with the pure compound used as standard (Table 3). *S. albus* GB16 + pLN2, when fed with 8DMTC, converted the aglycone to a compound consistent with LOVL-TCMC in terms of HPLC mobility and absorption spectrum when compared with the pure compound used as standard (Table 3). MALDI-TOF analysis of this

compound showed molecular peaks at m/z 611.0815 and 627.0728 for the sodium and potassium adducts of LOVL-TCMC, respectively (Table 3). The reporter system providing endogenous 8DMTC (*S. albus* 16F4) showed a greater efficiency of conversion to LOVL-TCMC (64%) compared to the biotransformation system (*S. albus* GB16) with only 12% conversion of the aglycone observed.

*S. lividans* NAG2 + pLN2 was found to convert 50% of supplied EB to a compound with HPLC mobility, absorption spectrum and LCMS analysis consistent with L-oleandrosyl-erythronolide B (LOLE-EB) as compared with a pure standard.

*S. lividans* NABV + pLN2 was found to convert supplied EB to a compound with HPLC mobility, absorption spectrum and LCMS analysis consistent with L-oleandrosyl-erythronolide B (LOLE-EB) as compared with a pure standard.

Example 3: Screening of heterologous 4-ketoreductases in pLN2 acting on L-6-deoxysugar intermediates

The 4-ketoreductase encoded by *oleU* was replaced, as described in the Material and Methods above and Figure 8, with one of the following heterologous 4-ketoreductases *eryBIV* or *snogC* of the erythromycin and nogalamycin clusters, respectively (Table 1). These reductases act on different sugar intermediates to OleU, but share the same C-4 ketoreductase stereospecificity.

Replacement of *oleU* with *eryBIV* (pLN2 derivative pLNBIV) gave rise to a new HPLC peak in both reporter systems *S. albus* 16F4 and *S. albus* GB16 (described in Example 1), with the conversion of 8DMTC at levels of 20% and 3% respectively. This new compound was consistent with LOLV-TCMC in terms of HPLC mobility and absorption spectrum when compared with the pure compound used as standard (Table 3). MALDI-TOF analysis of this compound showed molecular peaks at m/z 611.0815 and 627.0728 for the sodium and potassium adducts of LOLV-TCMC, respectively (Fig. 10).

No glycosylation of 8DMTC was observed on replacement of *oleU* with *snogC* (pLN2 derivative pLNS). The expected products of SnogC, L-olivose or L-rhamnose, are readily transferred to 8DMTC by ElmGT, lack of glycosylation indicates a narrow sugar substrate specificity of the *snogC* gene product.

Replacement of *oleU* with *eryBIV* (pLN2 derivative pLNBIV) gave rise to two new HPLC peaks in the reporter system *S. lividans* NAG2 fed with EB. These new compounds were consistent with a minor peak of LOLE-EB and a major peak of L-digitoxosyl-EB (LDIG-EB) in terms of HPLC mobility and absorption spectrum when compared with the pure compound used as standard. LCMS analysis of LDIG-EB showed molecular peaks at m/z 555.0 and 571.5 for the sodium and potassium adducts, respectively (Fig. 10A).

Replacement of *oleU* with *eryBIV* (pLN2 derivative pLNBIV) gave rise to three new HPLC peaks in the reporter system *S. lividans* NAB5 fed with EB. These new compounds were consistent with two minor peaks corresponding to LOLE-EB and L-3-methyl-digitoxosyl-EB (L3MDIG-EB) and a major peak of L-digitoxosyl-EB (LDIG-EB) in terms of HPLC mobility, absorption spectrum and LCMS analysis when compared with the pure compound used as standard.

Example 4: Screening of heterologous 4-ketoreductases in pLN2 acting on D-6-deoxysugar intermediates

The 4-ketoreductase TylD acts on D-6-deoxysugar intermediates but has the same stereospecificity at C-4 as OleU (Table 1). No glycosylation of 8DMTC was observed on replacement of *oleU* with *tylD* (pLN2 derivative pLNT) in the *S. albus* or *S. lividans* host strain reporter systems.

Example 5: Biosynthesis of L-rhamnose by two-gene deletion of *oleV* and *oleW* (pLN2 derivative pLN2Δ)

L-Rhamnose differs from L-olivose in containing an hydroxyl group at C-2. The cassette design of pLN2 enables the removal of the deoxygenation genes *oleV* and *oleW*, creating a pLN2 derivative pLN2Δ. The genes *oleV* and *oleW* were removed by digestion of pLN2 with *Avr*II and *Spe*I (generating compatible cohesive ends) and further re-ligation. The resulting construct should direct the biosynthesis of L-rhamnose (Fig. 6 and Fig. 11).

The construct, pLN2Δ, gave rise to a new glycosylated derivative in both reporter systems *S. albus* 16F4 and *S. albus* GB16 (described in Example 1). Once ElmGT transferred L-rhamnose onto the aglycone 8DMTC, the three O-methyltransferases present in cos16F4 methylated the three free hydroxyl groups of L-rhamnose with the formation of elloramycin

in *S. albus* 16F4 (Fig. 12). This new compound was consistent with elloramycin in terms of HPLC mobility and absorption spectrum when compared with the pure compound used as standard. MALDI-TOF analysis, in the negative mode, of this compound showed a molecular peak at m/z 659.0380, corresponding to elloramycin.

In *S. albus* GB16, MALDI-TOF analysis showed molecular peaks at m/z 627.0641 and 643.0388, corresponding to the respective sodium and potassium adducts of L-rhamnose-tetracenamycin C (LRHA-TCMC).

The construct, pLN2Δ, gave rise to a new glycosylated derivative in the reporter system *S. lividans* NAG2. This new compound was consistent with L-rhamnosyl-EB (LRHA-EB) in terms of HPLC mobility and absorption spectrum when compared with the pure compound used as standard. LCMSanalysis of this compound showed molecular peaks at m/z 571.5 and 588.0, corresponding to the sodium and potassium adducts of LRHA-EB (Fig. 12A).

Example 6: Biosynthesis of L-rhamnose by two-gene deletion of *oleV* and *oleW* (pLNBIV derivative pLNBIVΔ)

L-Rhamnose differs from L-olivose in containing an hydroxyl group at C-2. The cassette design of pLNBIV enables the removal of the deoxygenation genes *oleV* and *oleW*, creating a pLNBIV derivative pLNBIVΔ. The resulting construct should direct the biosynthesis of L-rhamnose (Fig. 6).

No glycosylated products were formed, demonstrating that EryBIV requires a C-2-deoxy sugar intermediate, whereas OleU has a broader substrate specificity acting on both 2,6-dideoxy and C-2-deoxy sugar intermediates (Example 3).

Example 7: Biosynthesis of L-rhodinose (pLN2 derivative pLNRHO)

L-olivose (2,6-dideoxysugar) and L-rhodinose (2,3,6-trideoxysugar with an equatorial hydroxyl group at C-4) are 6-deoxyhexoses that differ in the hydroxyl groups at C-3 and C-4.

In pLN2, *oleU* was replaced with the 4-ketoreductase *urdZ3* creating pLNZ3. UrdZ3 participates in the biosynthesis of L-rhodinose, one of the sugars of the sugars forming part of urdamycin A. A second gene encoding a 3,4-dehydratase, *urdQ* from the same cluster, was inserted into pLNZ3 giving rise to the derivative pLNRHO.

The construct pLNRHO gave rise to a new HPLC peak in both reporter systems *S. albus* 16F4 and *S. albus* GB16 (described in Example 1). This new compound was consistent with L-rhodinosyl-tetracenomycin C (LRHO-TCMC) in terms of HPLC mobility and absorption spectrum when compared with the pure compound used as standard (Table 3). MALDI-TOF analysis of this compound, in the negative mode, showed a molecular peak at m/z 571.0760, consistent with LRHO-TCMC.

The construct pLNRHO gave rise to a new HPLC peak in the reporter system *S. lividans* NAB5 fed with EB. This new compound was consistent with a peak corresponding to L-rhodinosyl-EB (LRHO-EB) in terms of HPLC mobility, absorption spectrum and LCMS analysis when compared with the pure compounds used as standard.

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**Table 1. Genes used for the construction of cassette plasmids**

| Gene | Microorganism | Antibiotic | Sugar pathway | Proposed function | Reference |
|---|---|---|---|---|---|
| *oleS* | *S.antibioticus* | Oleandomycin | L-oleandrose and D-desosamine | Glucose synthase | Agui., 2000 |
| *oleE* | *S.antibioticus* | Oleandomycin | L-oleandrose and D-desosamine | Glucose 4,6-dehydratase | Agui 2000 |
| *oleL* | *S.antibioticus* | Oleandomycin | L-oleandrose | 3,5-epimerase | Agui, 2000 |
| *oleV* | *S.antibioticus* | Oleandomycin | L-oleandrose | 2,3-dehydratase | Agui, 2000 |
| *oleW* | *S.antibioticus* | Oleandomycin | L-oleandrose | 3-ketoreductase | Agui 2000 |
| *oleU* | *S.antibioticus* | Oleandomycin | L-oleandrose | 4-ketoreductase | Agui2000 |
| *oleY* | *S.antibioticus* | Oleandomycin | L-oleandrose | 3-O-methyltransferase | Olano,1998, Rodriguez 2001 |
| *eryBIV* | *Sacc. erythraea* | Erythromycin | L-mycarose | 4-ketoreductase | Gaisser Summer |
| *snogC* | *S.nogalater* | Nogalamycin | L-nogalose | 4-ketoreductase | AAF01815 |
| *dnmV* | *S.peucetius* | Daunorubicin | L-daunosamine | 4-ketoreductase | Otten |
| *tylD* | *S.fradiae* | Tylosin | 6-deoxy-D-allose | 4-ketoreductase | Fouces; Bate |

Table 2. Synthetic oligonucleotides used for PCR amplification of individual sugar biosynthetic genes

| Primers | *Gene* | DNA sequence (5'-3') | Restriction sites |
|---|---|---|---|
| 5'-7U | *oleU* | AGAG**AAGCTTACTAGT**GCCCCCCCGAGAGGC AGCGGCCCATG | *Hind*III, *Spe*I |
| 3'-7L | | AG**TCTAGAGCTAGC**TCATGCTGCTCCTCGCCG GGTCGGTGGG | *Xba*I, *Nhe*I |
| 5'-14U | *oleY* | AGAG**AAGCTTGCTAGC**CGGACCACGCGAAGG ACCTTTCACATG | *Hind*III, *Nhe*I |
| 3'-14L | | AG**TCTAGATTAATTAA**GCTAGTTGTCGTTCCA GAACGGCTCCCGG | *Xba*I, *Pac*I |
| 5'-1α | *oleV* | ACGT**AAGCTTCCTAGG**GCGCCGTCCTGGATCA CAATG | *Hind*III, *Avr*II |
| 3'-1β | | ACG**TCTAGAGTTAAC**CTTCGGGGTGCTCAGCT CAGG | *Xba*I, *Hpa*I |
| 5'-15α | *oleW* | ACG**TAAGCTTGTTAAC**CCGAAGGGAACCCCAT GCCC | *Hind*III, *Hpa*I |
| 3'-15β | | ACG**TCTAGAACTAGT**GCATCAGCACCAGCGCA CCCG | *Xba*I, *Spe*I |
| 5'-VU | *dnmV* | AGAG**AAGCTTACTAGT**GGGTCACGCGGAGAC GGGTGAGGCGGACATG | *Hind*III, *Spe*I |
| 3'-VL | | AG**TCTAGAGCTAGC**CTAGGCCGGGGCGCCGT GCGCGGTCC | *Xba*I, *Nhe*I |
| 5'-SU | *snogC* | AGAG**AAGCTTACTAGT**GCGTGCTGGTCACCG GAGGAGAAGGCATG | *Hind*III, *Spe*I |
| 3'-SL | | AG**TCTAGAGCTAGC**TCACGTCCCCGTCAGGTT CCTGAGGCGC | *Xba*I, *Nhe*I |
| 5'-TU | *tylD* | AGAG**AAGCTTACTAGT**CGCTCCGCCCACCGG GCGCGACGCATG | *Hind*III, *Spe*I |
| 3'-TL | | AG**TCTAGAGCTAGC**TCACGAGTGGGGTGCCC GCGACGGCCGG | *Xba*I, *Nhe*I |
| 5'-B4U | *eryBIV* | AGAG**AAGCTTACTAGT**CAGTGACGGGTTGCC GCACATCGCGCTGAATG | *Hind*III, *Spe*I |
| 3'-B4L | | AG**TCTAGAGCTAGC**CTAGTGCTCCTCGGTGG GGGTCAGGGCGGCC | *Xba*I, *Nhe*I |

**Table 3. Plasmid constructs directing the biosynthesis of different sugars and GTF system used to show the presence of the sugar**

| Plasmid | *oleU* replaced by | Sugar expected | GTF used | | |
|---|---|---|---|---|---|
| | | | *oleG2* | *eryBV* | *elmGT* |
| pLN2 | ----- | L-olivose (L-oleandrose) | L-oleandrosyl-EB | L-oleandrosyl-EB | L-olivosyl-TcmC |
| pLN2EryBIV | *eryBIV* | L-olivose (L-oleandrose) | L-oleandrosyl-EB | L-oleandrosyl-EB* L-digitoxyl-EB 3-O-methyl-L-digitoxyl-EB | L-digitoxyl-TcmC |
| pLN2SnogC | *snogC* | L-olivose (L-oleandrose) | ---- | ----- | ----- |
| pLN2DnmV | *dnmV* | L-oliose | ---- | ----- | ----- |
| pLN2TylD | *tylD* | Unknown | ---- | ----- | ----- |
| pLN2ΔVW | ----- | L-rhamnose | L-rhamnosyl-EB | ----- | L-rhamnosyl-TcmC |
| pLNRHO | *urdZ3; urdQ* | L-rhodinose | | L-rhodinose-EB | L-rhodinose-TcmC |
| pLNR | *urdR* | D-olivose (D-oleandrose) | | D-olivosyl-EB D-oleandrosyl-EB | D-olivosyl-TcmC |

**CLAIMS:**

1. A process for producing a hybrid glycosylated product by transferring one or more sugar moieties to an aglycone template, the process comprising:

transformation of microorganism host cells: (a) with a plasmid-based gene cassette which contains nucleic acid encoding genes sufficient to direct the synthesis of a specific activated sugar in those host cells; and (b) with nucleic acid encoding a glycosyltransferase (GT); and,

providing an aglycone template to the transformed microorganism, or allowing its endogenous generation, so that the GT transfers one or more sugar moieties to the aglycone template in order to produce a hybrid glycosylated product;

wherein one or more of the sugar moieties, the aglycone template, the glycosyltransferase, the sugar synthesis genes or the host cells are heterologous to one or more of the other components.

2. A process according to claim 1 wherein at least some of said sugar synthesis genes in the cassette are flanked by restriction sites.

3. A process according to claim 2 wherein each of said genes is flanked by restriction sites.

4. A process according to claim 2 or claim 3 wherein said genes, with their restriction sites if present, are generated by means of the polymerase chain reaction (PCR).

5. A process according to claim 4 wherein a multiplicity of sugar synthesis genes are generated by the PCR and combinatiorially assembled to produce a multiplicity of different cassettes which are used in the transformation of respective different host cells.

6. A process according to any preceding claim wherein at least some, and preferably all, of said sugar synthesis genes have their own ribosome binding sites.

7. A process according to any preceding claim wherein the aglycone template and the GT are mutually heterologous.

8. A process according to any preceding claim wherein the aglycone template is exogenously supplied.

9. A process according to any preceding claim wherein the aglycone template comprises a polyketide.

10. A process according to any preceding claim wherein the aglycone template comprises a peptide.

11. A process according to any preceding claim wherein said cassette comprises some or all of the genes required for the synthesis of one or more of oleandrose,

desosamine, mycarose, mycaminose, rhodinose, oliose and olivose.

12. A transformant host cell produced by transforming a precursor host cell with (a) a gene expression cassette which contains nucleic acid encoding genes sufficient to direct the synthesis of a specific activated sugar in those host cells, optionally operably linked under the same promoter; and (b) with a glycosyltransferase (GT), wherein the GT is heterologous to the host cells and is capable of transferring one or more sugar moieties to an aglycone template within the cells to produce a hybrid glycosylated product.

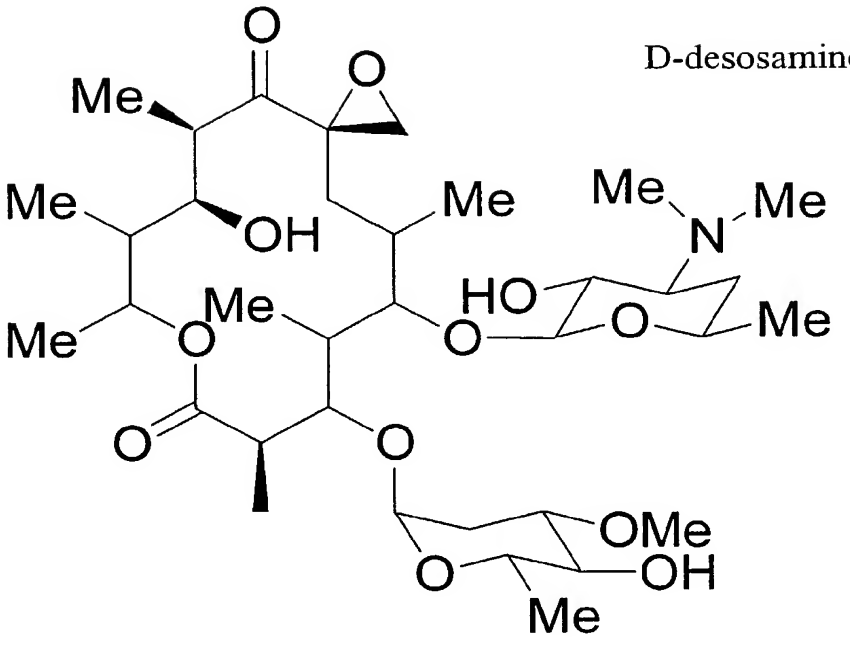
13. A transformant host cell according to claim 12 wherein said cassette comprises some or all of the genes required for the synthesis of one or more of oleandrose, desosamine, mycarose, mycaminose, rhodinose, oliose and olivose.

14. A process for producing a hybrid glycosylated product, the process comprising culturing the host cells according to claim 12 or claim 13 and isolating the product thus produced.

15. A process according to claim 14 including the additional step of supplying the aglycone template to the cells.

16. A hybrid glycosylation product as obtainable by any of the processes of claims 1-11, 14 or 15.

# Figure 1

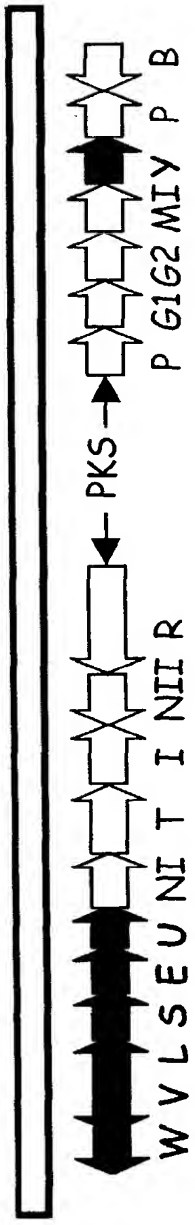



D-desosamine

L-oleandrose

Oleandomycin

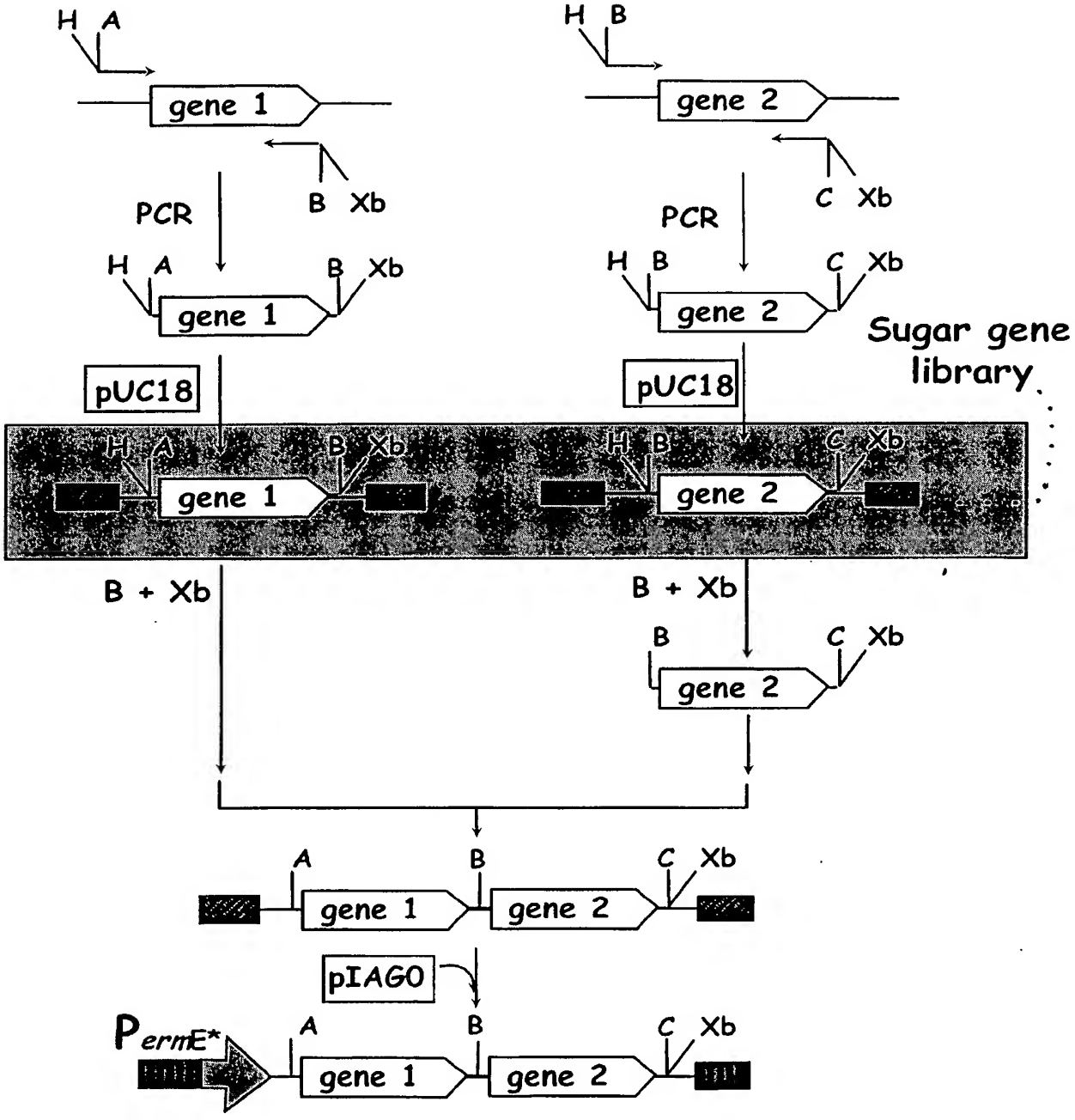
Figure 2




**Genes involved in the dTDP-L-oleandrose biosynthesis**

| | |
|---|---|
| *oleW* | 3-reductase |
| *oleV* | 2,3-dehydratase |
| *oleL* | 3,5-epimerase |
| *oleS* | glucose synthase |
| *oleU* | 4-ketoreductase |
| *oleE* | 4,6-dehydratase |
| *oleY* | 3-O-methyltransferase |

# Figure 3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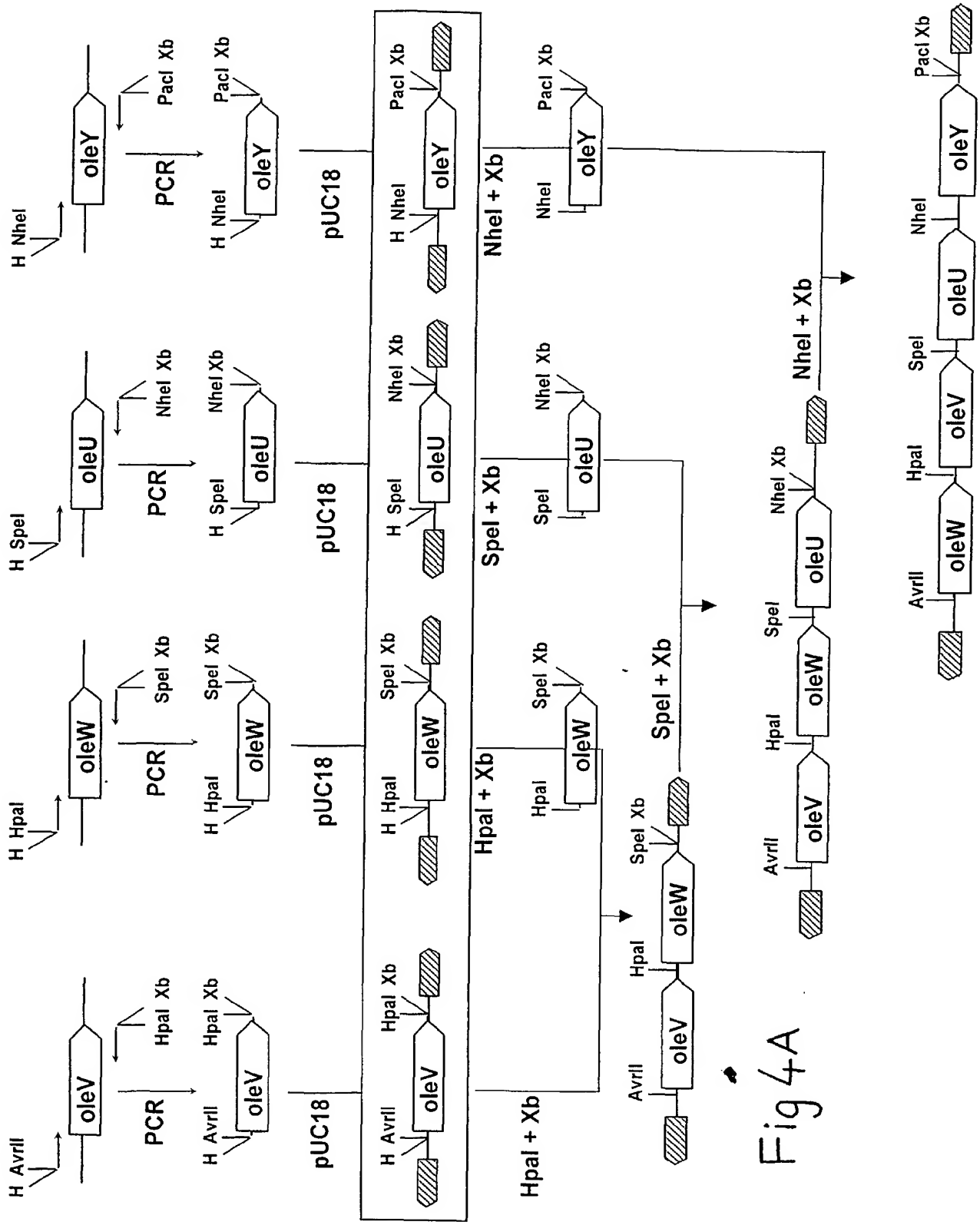
gene 1
B Xb
PCR
H A
B Xb
gene 1
pUC18
H B
gene 2
C Xb
PCR
H B
C Xb
gene 2
pUC18
Sugar gene library
H A
B Xb
gene 1
H B
C Xb
gene 2
B + Xb
B + Xb
B
C Xb
gene 2
A
B
C Xb
gene 1
gene 2
pIAGO
PermE*
A
B
C Xb
gene 1
gene 2


H NheI
oleY
PacI Xb
PCR
H NheI
oleY
PacI Xb
pUC18
H NheI
oleY
PacI Xb
NheI + Xb
NheI
oleY
PacI Xb
H SpeI
oleU
NheI Xb
PCR
H SpeI
oleU
NheI Xb
pUC18
H SpeI
oleU
NheI Xb
SpeI + Xb
SpeI
oleU
NheI Xb
H HpaI
oleW
SpeI Xb
PCR
H HpaI
oleW
SpeI Xb
pUC18
H HpaI
oleW
SpeI Xb
HpaI + Xb
HpaI
oleW
SpeI Xb
SpeI + Xb
H AvrII
oleV
HpaI Xb
PCR
H AvrII
oleV
HpaI Xb
pUC18
H AvrII
oleV
HpaI Xb
HpaI + Xb
AvrII
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SpeI Xb
AvrII
oleV
HpaI
oleW
SpeI
oleU
NheI Xb
NheI + Xb
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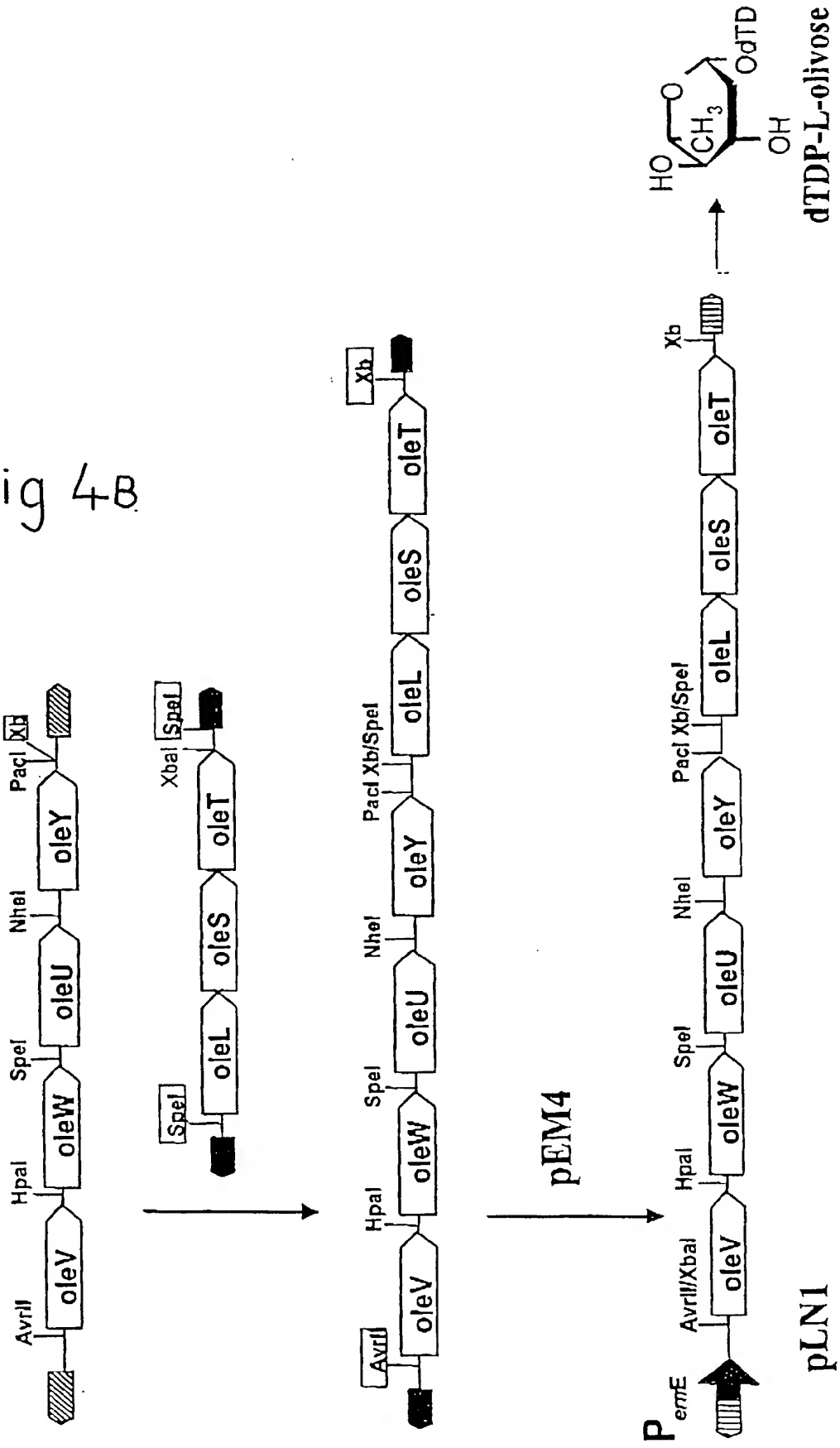
oleW
HpaI
oleV
SpeI
oleU
NheI
oleY
PacI Xb

Fig 4A

Fig 4B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SpeI
oleW
NheI
oleU
PacI
XbaI
oleY
SpeI
oleL
oleS
oleT
pEM4
pLN1
P ermE
dTDP-L-olivose
HO
CH3
O
OdTD
OH

# Figure 5

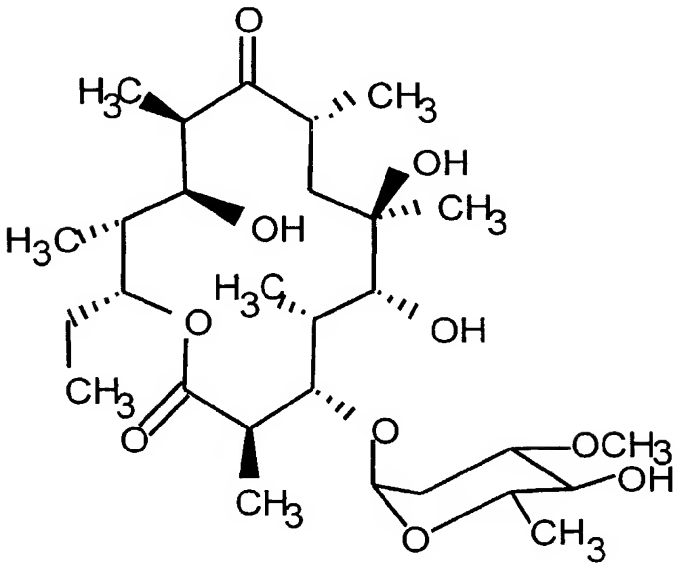

1- Erythronolide B

2- NAG2 + pOLE

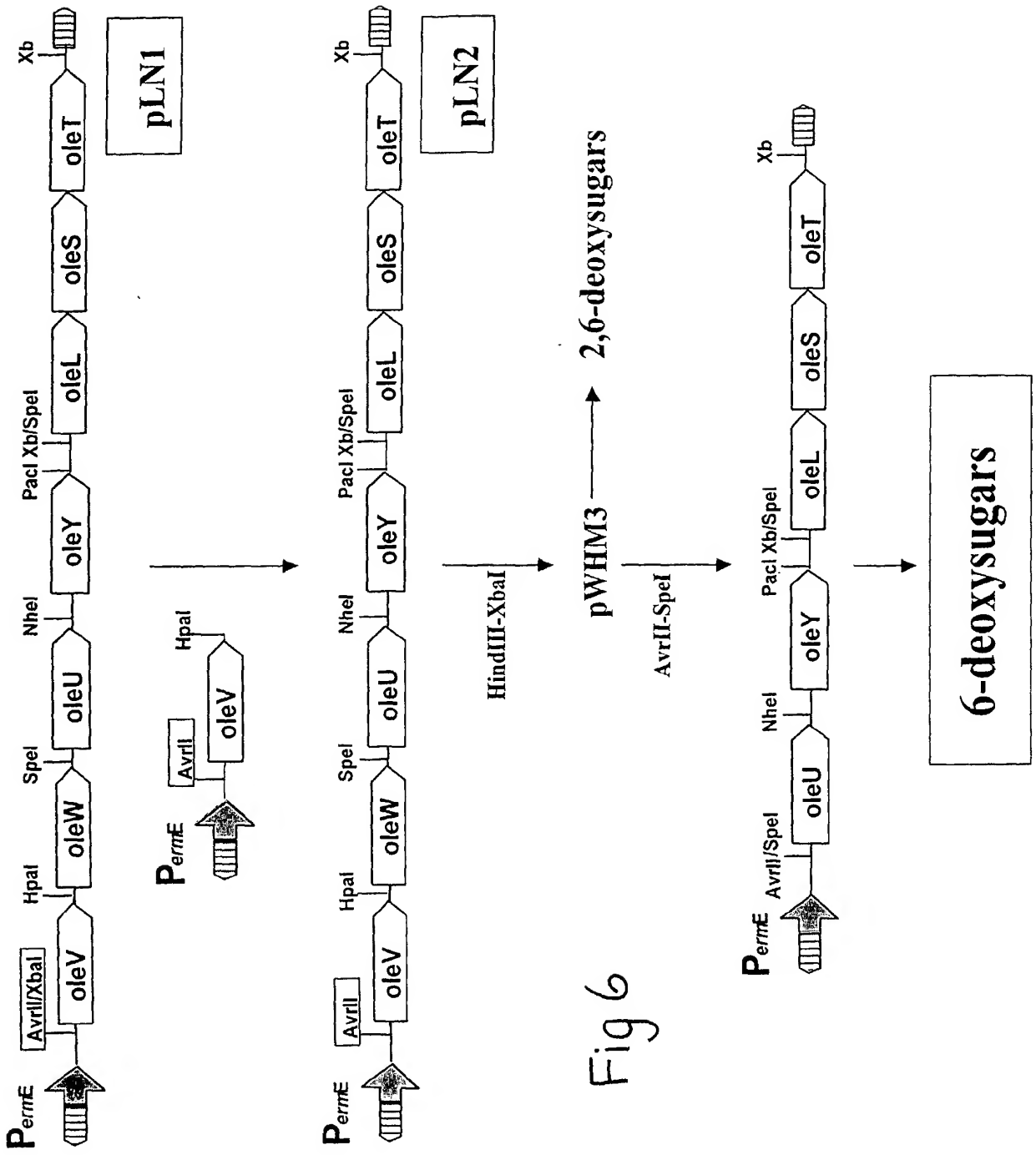
3- NAG2 + pOLV

4- NAG2 + pLN2

5- oleandrosyl-EB




Oleandrosyl-erythronolide B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oleV
HpaI
oleW
SpeI
oleU
NheI
oleY
PacI Xb/SpeI
oleL
oleS
oleT
Xb
pLN1
PermE
AvrII
HpaI
oleV
HindIII-XbaI
pWHM3
2,6-deoxysugars
AvrII-SpeI
PermE
AvrII/SpeI
oleU
NheI
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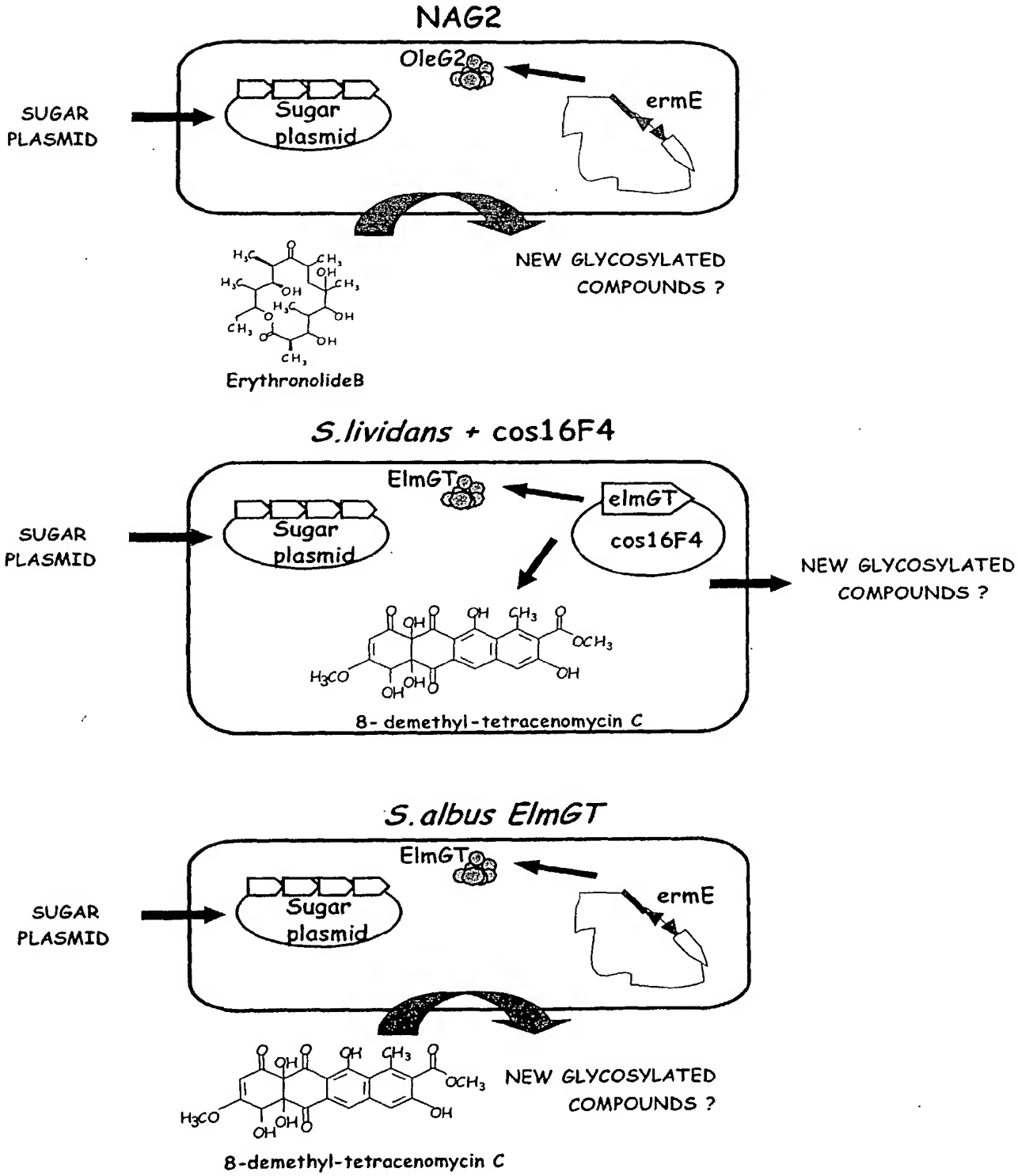
oleS
oleT
Xb
6-deoxysugars

Fig 6

# Figure 7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OleG2
ermE
SUGAR PLASMID
Sugar plasmid
NEW GLYCOSYLATED COMPOUNDS ?
ErythronolideB
S.lividans + cos16F4
ElmGT
elmGT
cos16F4
SUGAR PLASMID
Sugar plasmid
NEW GLYCOSYLATED COMPOUNDS ?
8- demethyl-tetracenomycin C
S.albus ElmGT
ElmGT
ermE
SUGAR PLASMID
Sugar plasmid
NEW GLYCOSYLATED COMPOUNDS ?
8-demethyl-tetracenomycin C

Figure 8

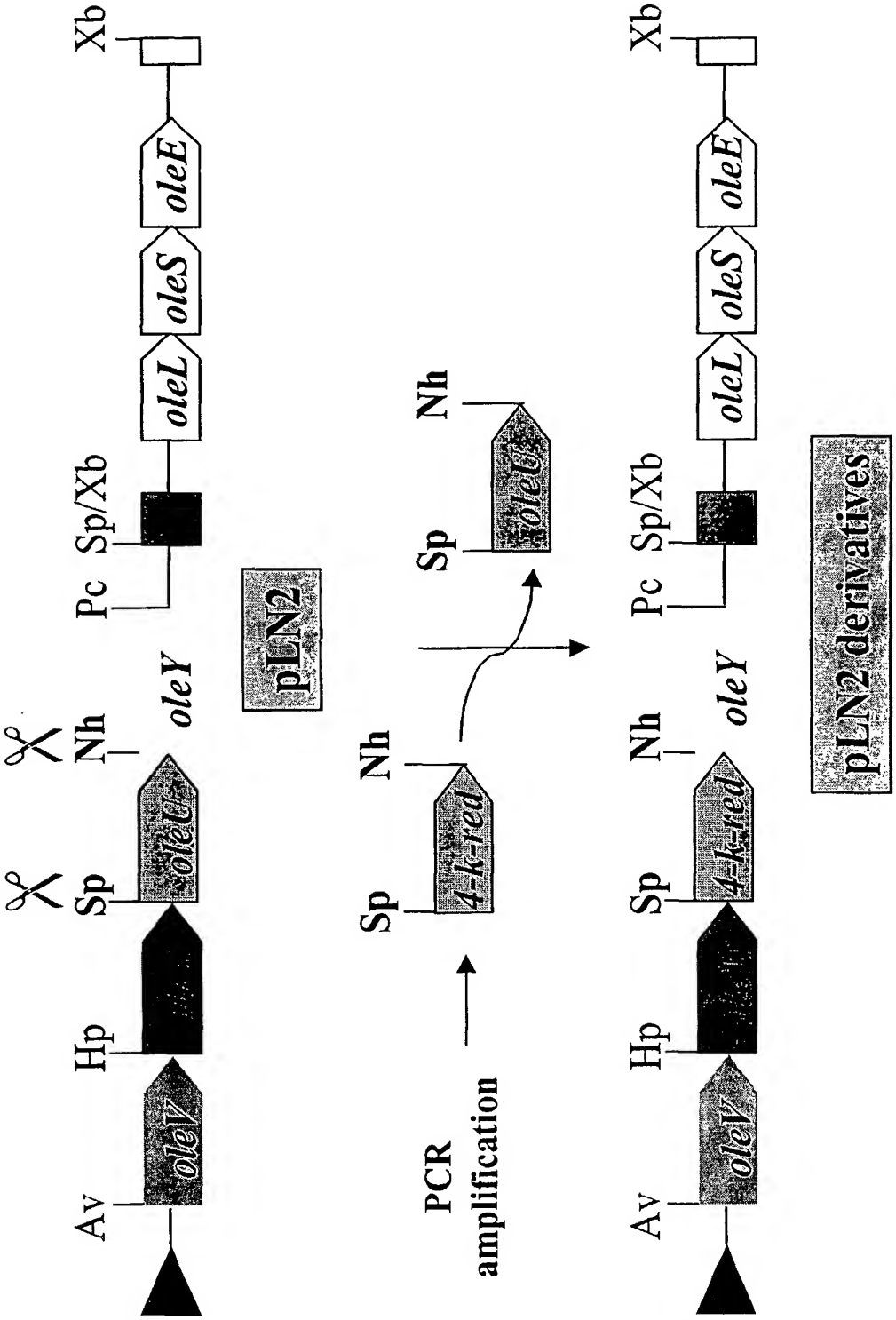
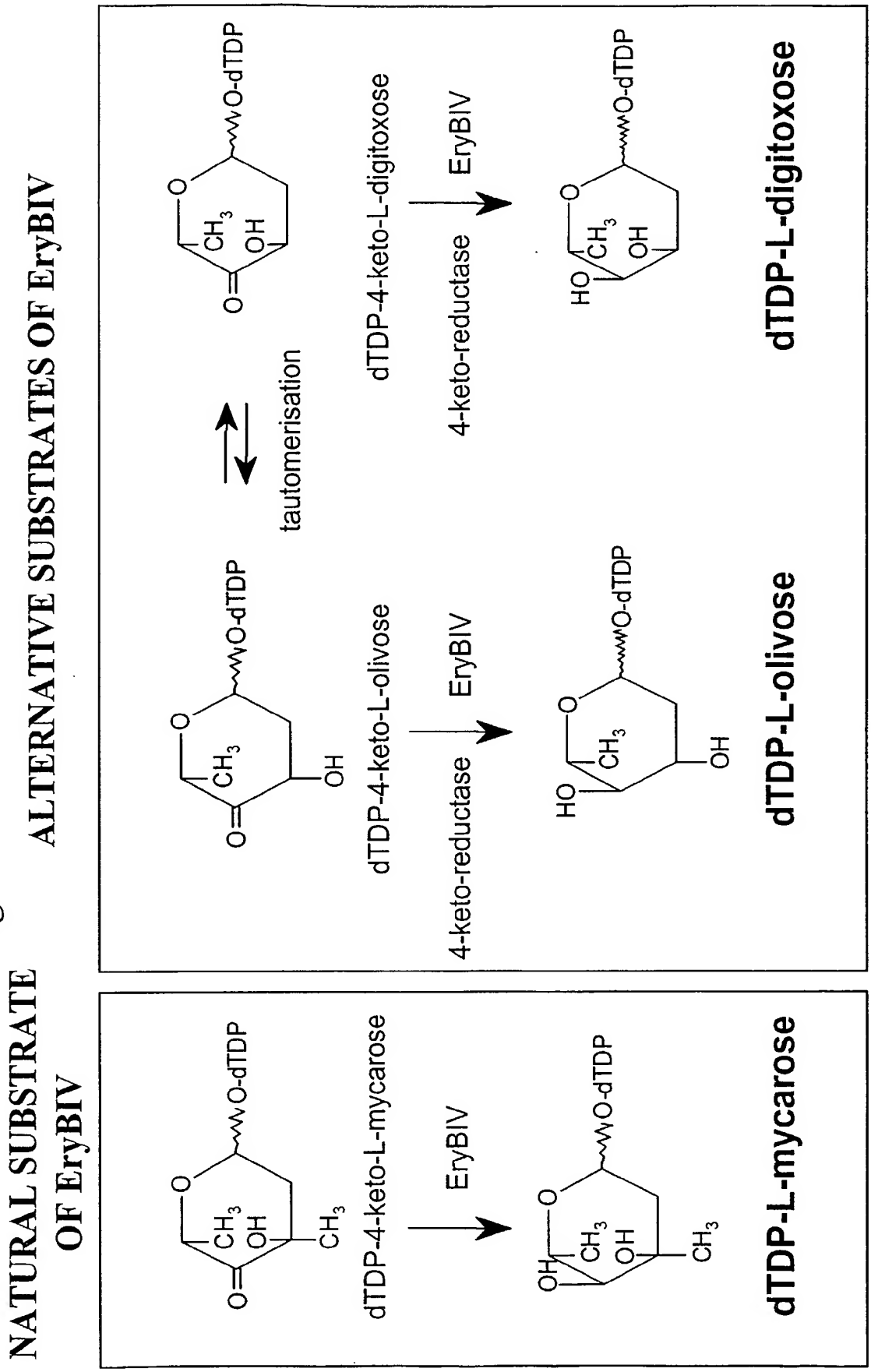

Av
oleV
Hp
Sp
oleG
Nh
oleY
Pc
Sp/Xb
oleL
oleS
oleE
Xb
pLN2
PCR amplification
4-k-red
pLN2 derivatives

Figure 9


NATURAL SUBSTRATE OF EryBIV
ALTERNATIVE SUBSTRATES OF EryBIV
dTDP-4-keto-L-mycarose
EryBIV
dTDP-L-mycarose
dTDP-4-keto-L-olivose
4-keto-reductase
EryBIV
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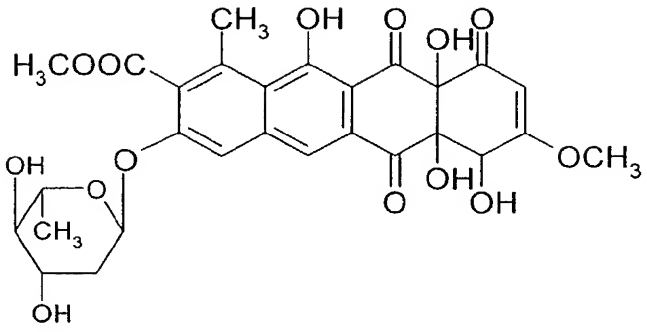
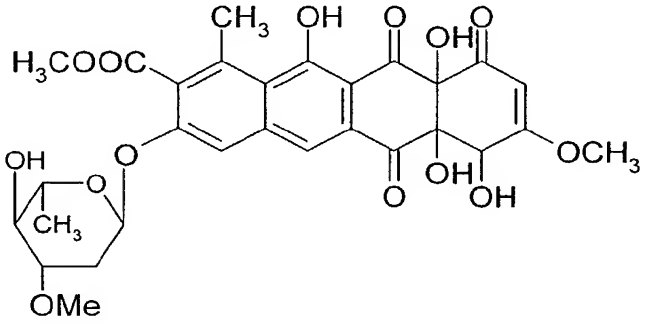
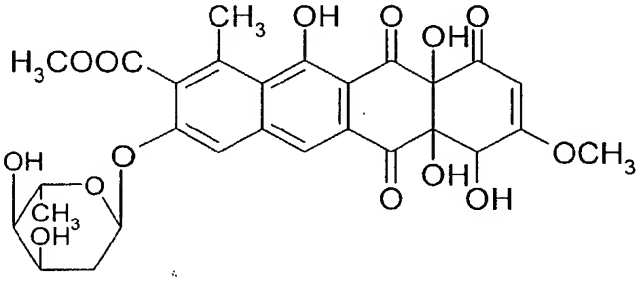



L-olivosyl-8-demethyl-tetracenomycin C

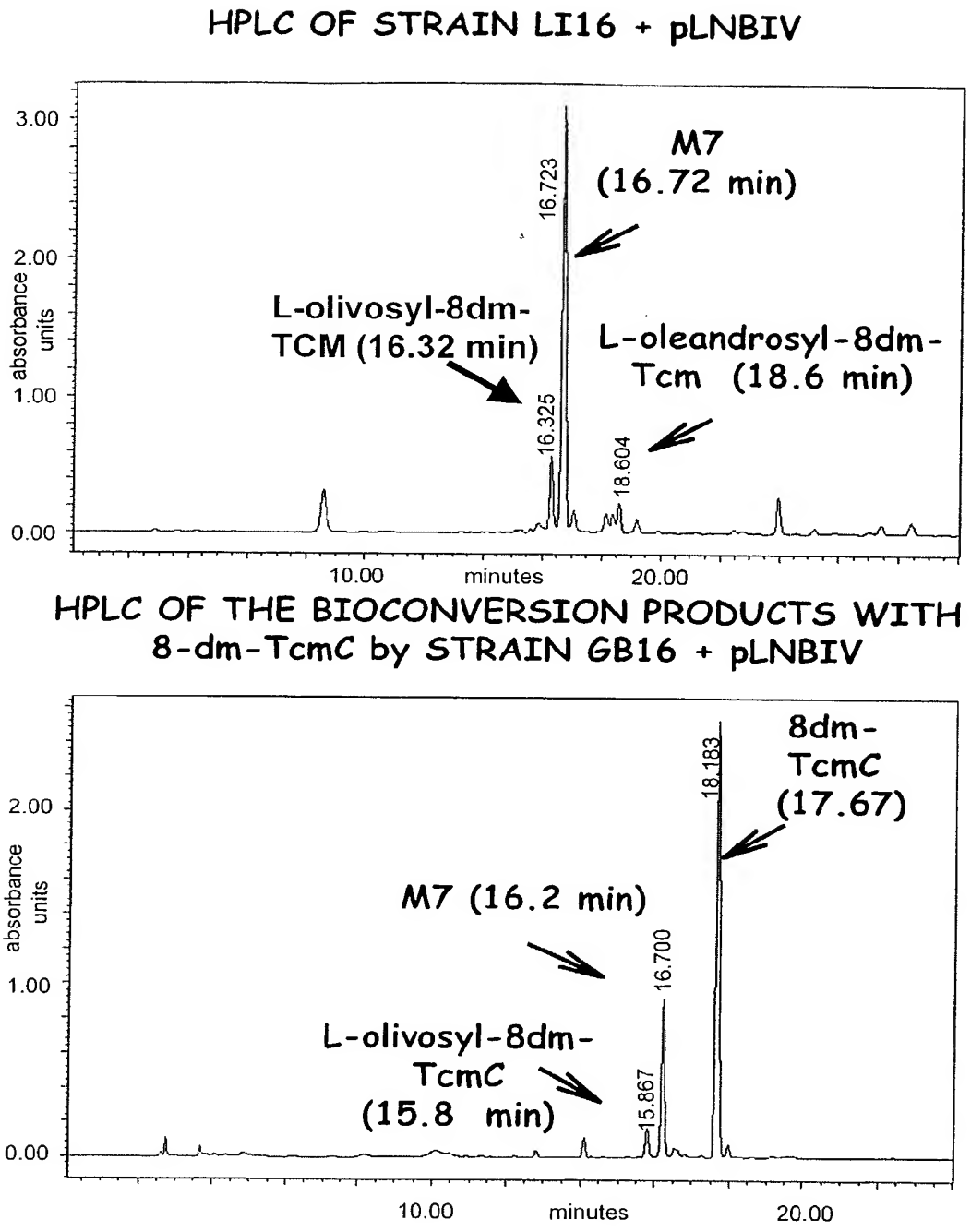



L-oleandrosyl-8-demethyl-tetracenomycin C




L-digitoxyl-8-demethyl-tetracenomycin C

Fig 10a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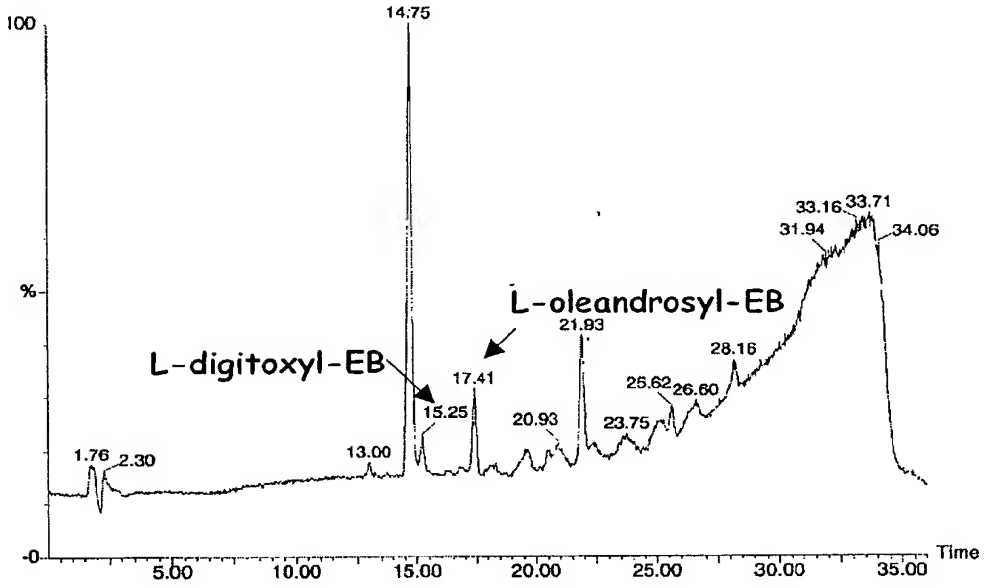
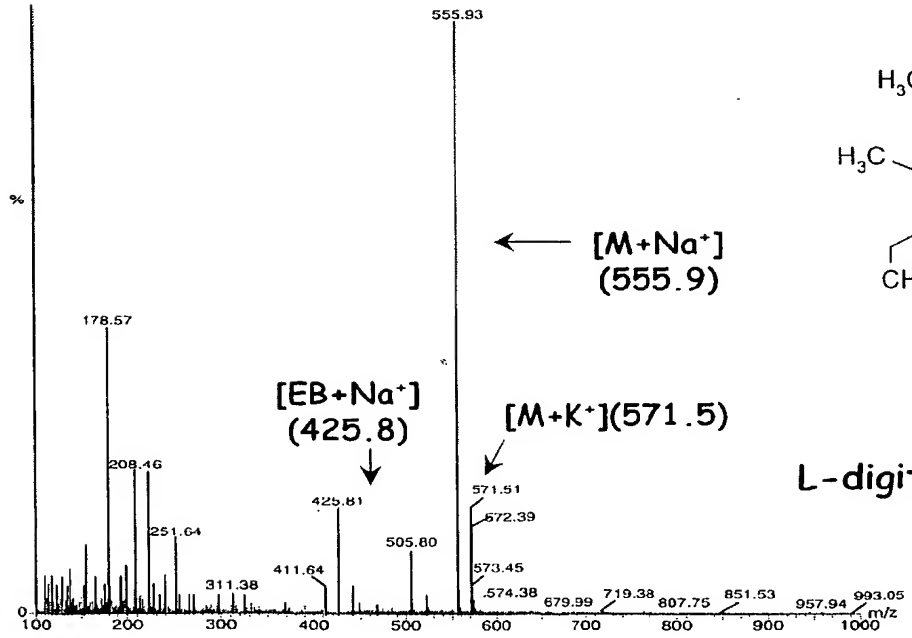
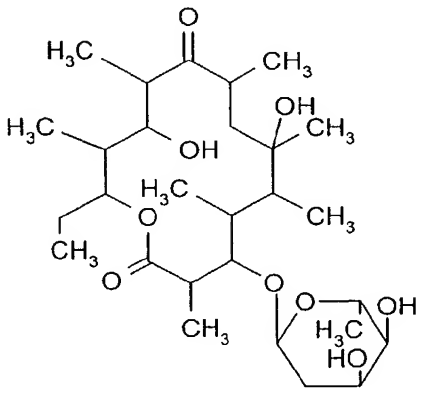
2.00
1.00
0.00
absorbance units
16.723
M7
(16.72 min)
L-olivosyl-8dm-
TCM (16.32 min)
16.325
L-oleandrosyl-8dm-
Tcm (18.6 min)
18.604
10.00
minutes
20.00
HPLC OF THE BIOCONVERSION PRODUCTS WITH
8-dm-TcmC by STRAIN GB16 + pLNBIV
2.00
1.00
0.00
absorbance units
18.183
8dm-
TcmC
(17.67)
M7 (16.2 min)
16.700
L-olivosyl-8dm-
TcmC
(15.8 min)
15.867
10.00
minutes
20.00

Fig 10b

# HPLC AND HPLC-MS ANALYSIS OF THE BIOCONVERSION PRODUCTS OF STRAIN NAG2+pLNBIV WITH EB



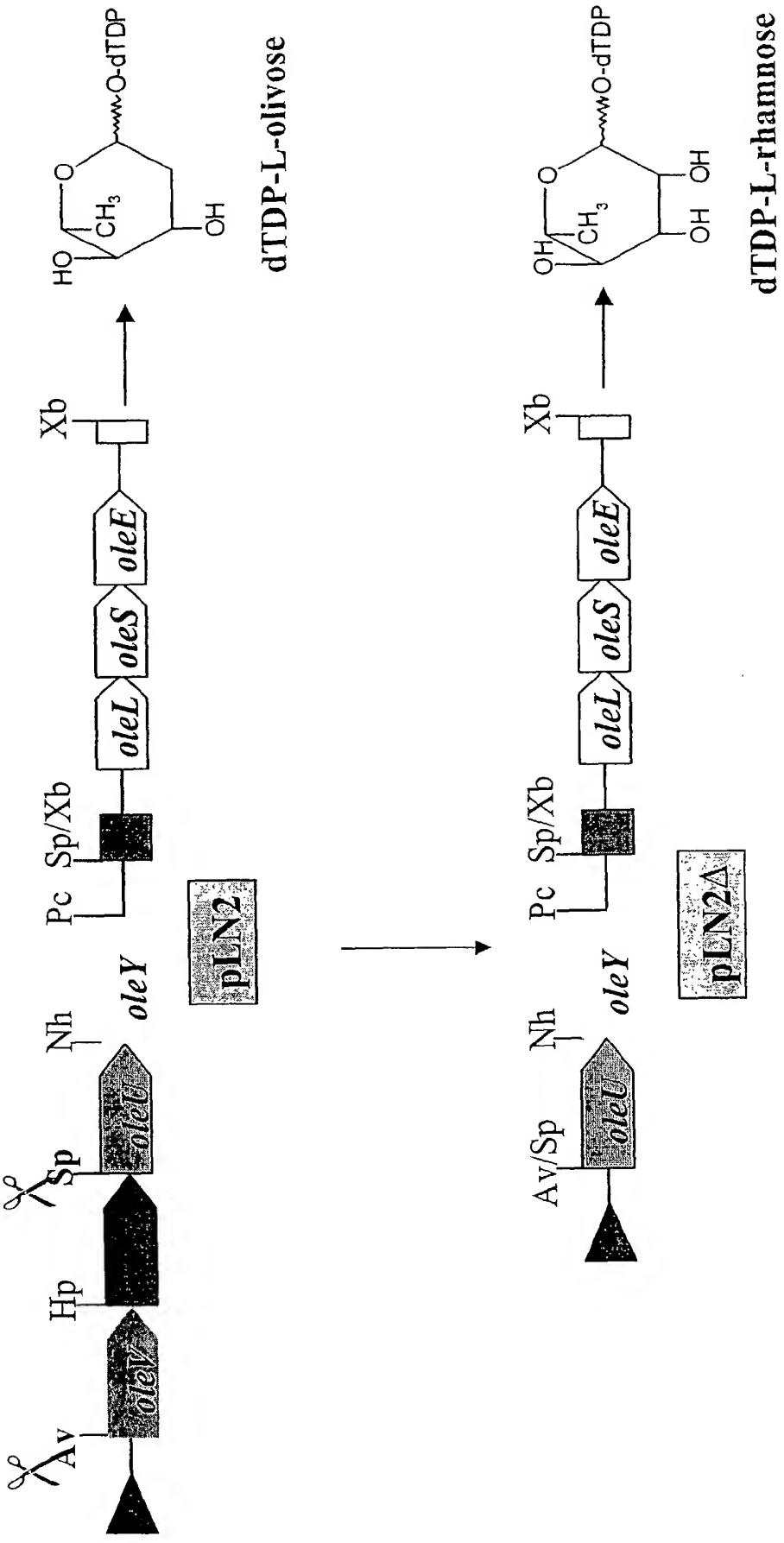




L-digitoxyl-erythronolide B

Fig 10A

Figure 11


Av
oleV
Hp
Sp
oleC
Nh
oleY
Pc
Sp/Xb
oleL
oleS
oleE
Xb
pLN2
dTDP-L-olivose
HO
CH3
O
O-dTDP
OH
Av/Sp
oleU
pLN2Δ
dTDP-L-rhamnose
OH
OH
OH

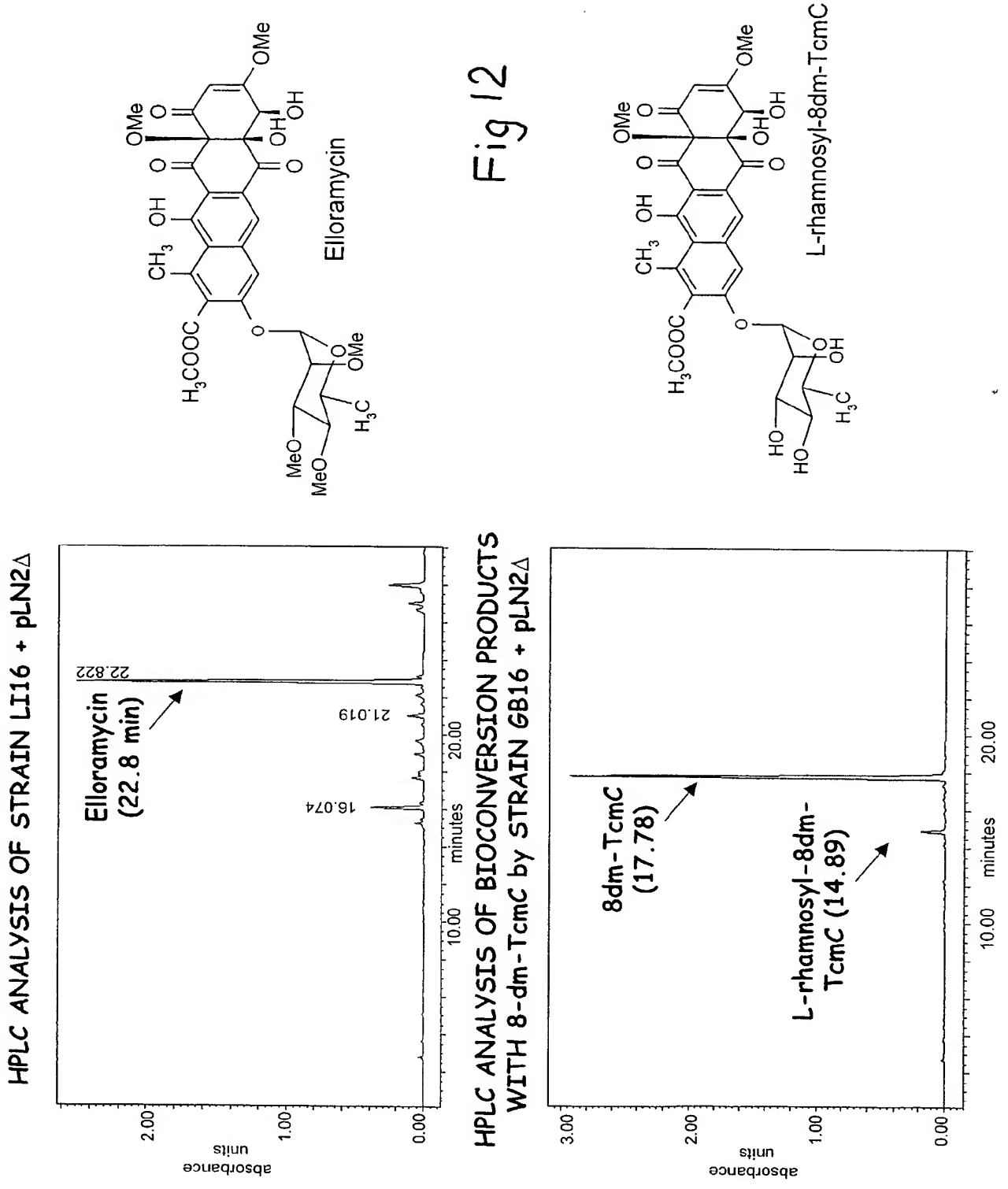
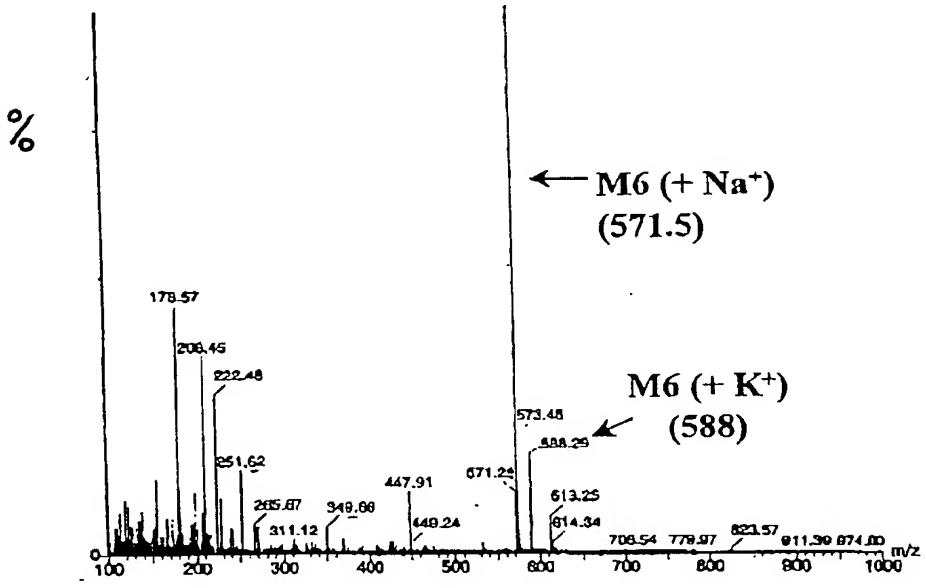

HPLC ANALYSIS OF STRAIN LI16 + pLN2Δ
Elloramycin (22.8 min)
22.822
21.019
16.074
absorbance units
2.00
1.00
0.00
10.00
minutes
20.00
Elloramycin
HPLC ANALYSIS OF BIOCONVERSION PRODUCTS WITH 8-dm-TcmC by STRAIN GB16 + pLN2Δ
8dm-TcmC (17.78)
L-rhamnosyl-8dm-TcmC (14.89)
3.00
2.00
1.00
0.00
absorbance units
10.00
minutes
20.00
L-rhamnosyl-8dm-TcmC

Fig 12

**HPLC ANALYSIS OF THE BIOCONVERSION PRODUCTS OF THE STRAIN NAG2 + pLN2Δ WITH EB**



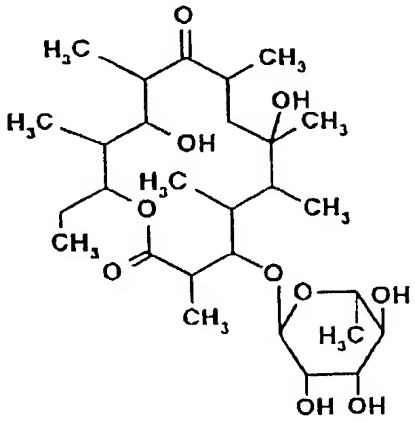




Fig 12a

L-rhamnosyl-erythronolide B

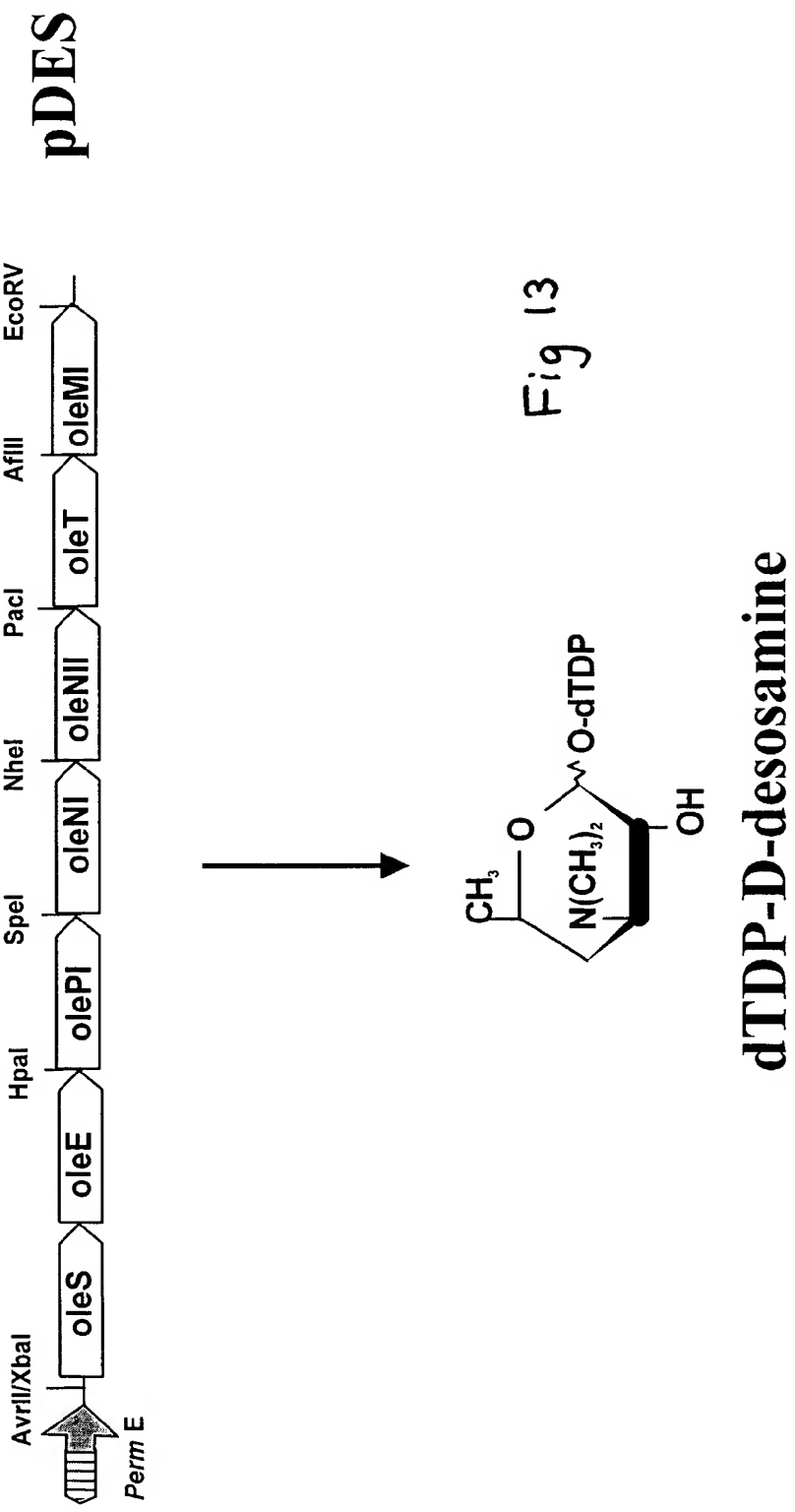

pDES
AvrII/XbaI
oleS
oleE
HpaI
olePI
SpeI
oleNI
NheI
oleNII
PacI
oleT
AflII
oleMII
EcoRV
Perm E
CH3
O
O-dTDP
N(CH3)2
OH
dTDP-D-desosamine

Fig 13

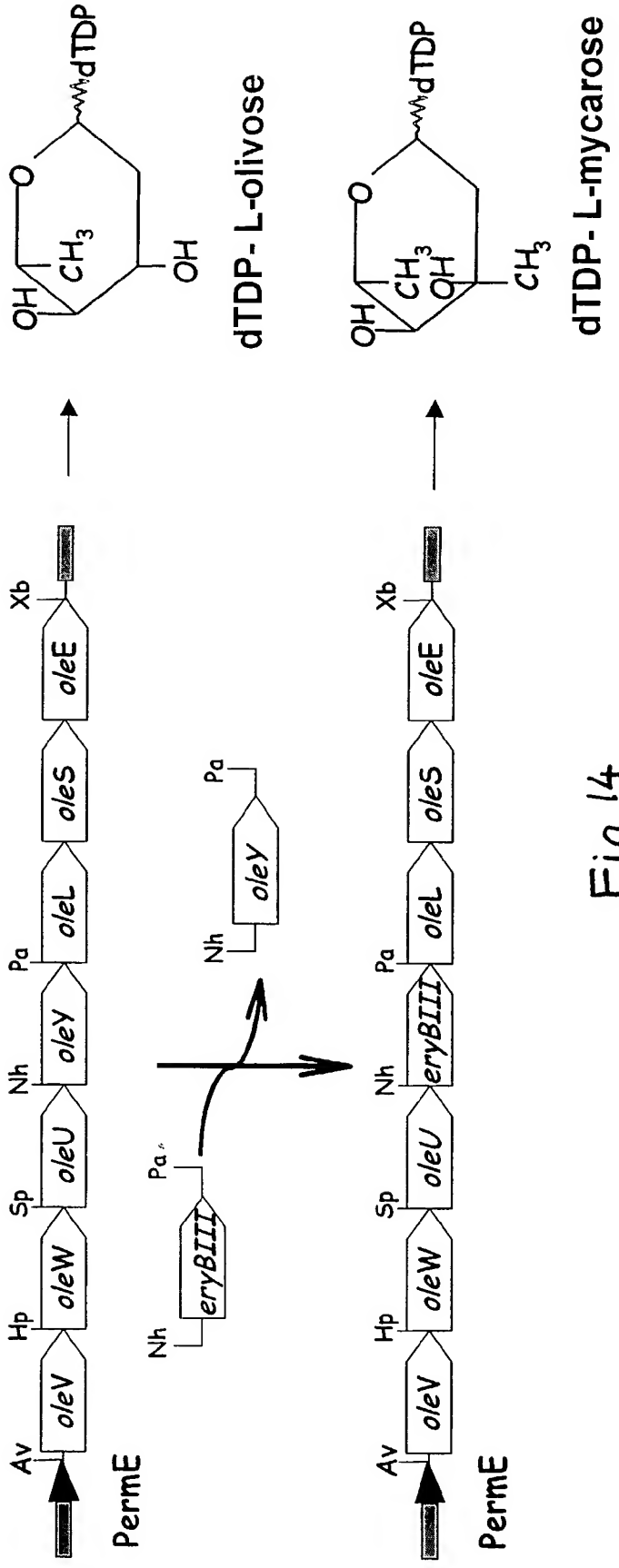

dTDP
O
CH3
OH
OH
dTDP- L-olivose
dTDP
O
CH3
OH
OH
CH3
dTDP- L-mycarose
Xb
oleE
oleS
oleL
Pa
oleY
Nh
oleU
Sp
oleW
Hp
oleV
Av
PermE
Pa
oleY
Nh
eryBIII
Pa
Nh
eryBIII
Pa
oleL
oleS
oleE
Xb
oleV
Av
oleW
Hp
oleU
Sp
Nh
PermE

Fig 14

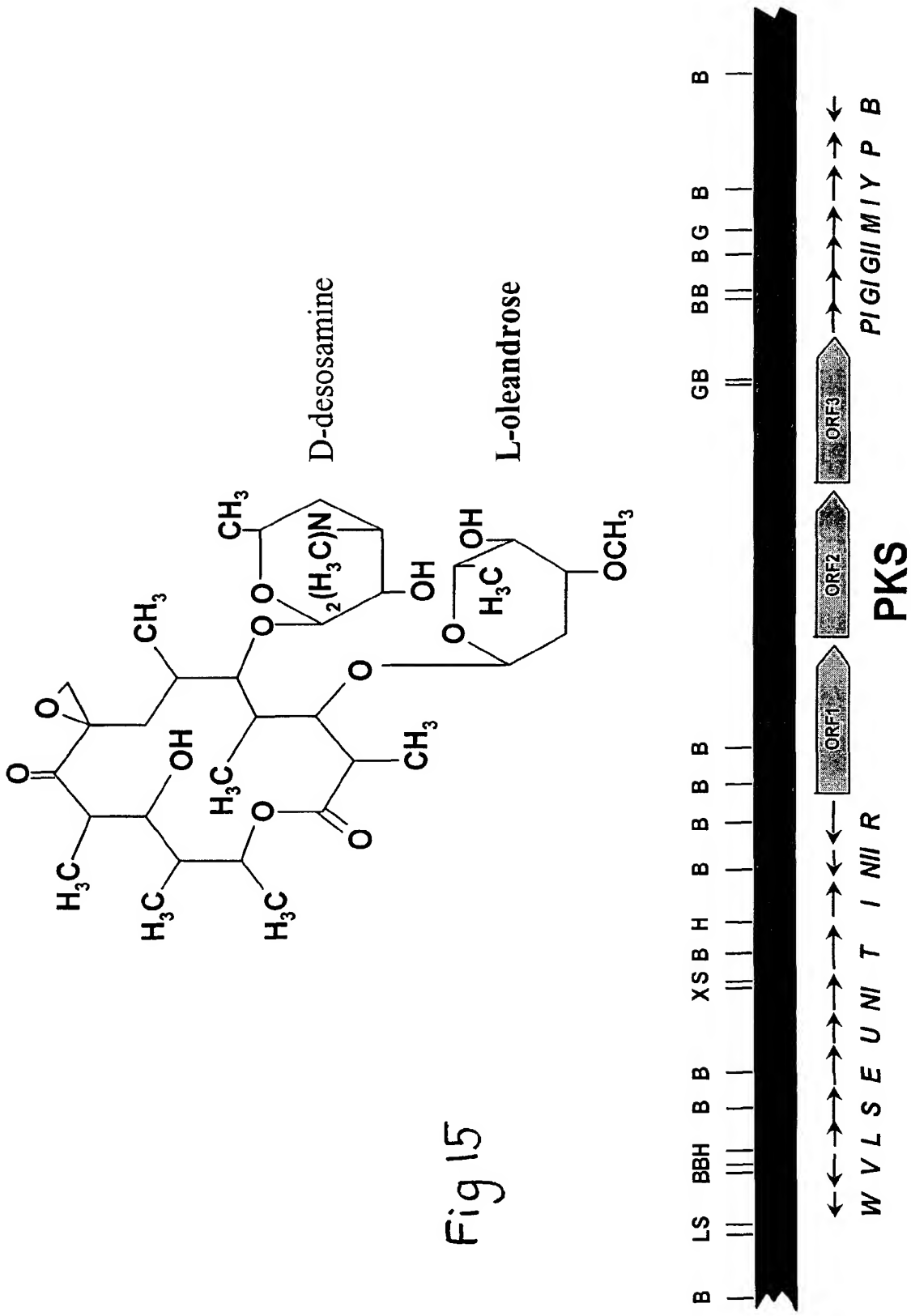

D-desosamine
L-oleandrose
B LS BBH B B XS B H B B B B B
GB BB B G B B
ORF1
ORF2
ORF3
PKS
W V L S E U N I T I N II R
P I G II M I Y P B

Fig 15

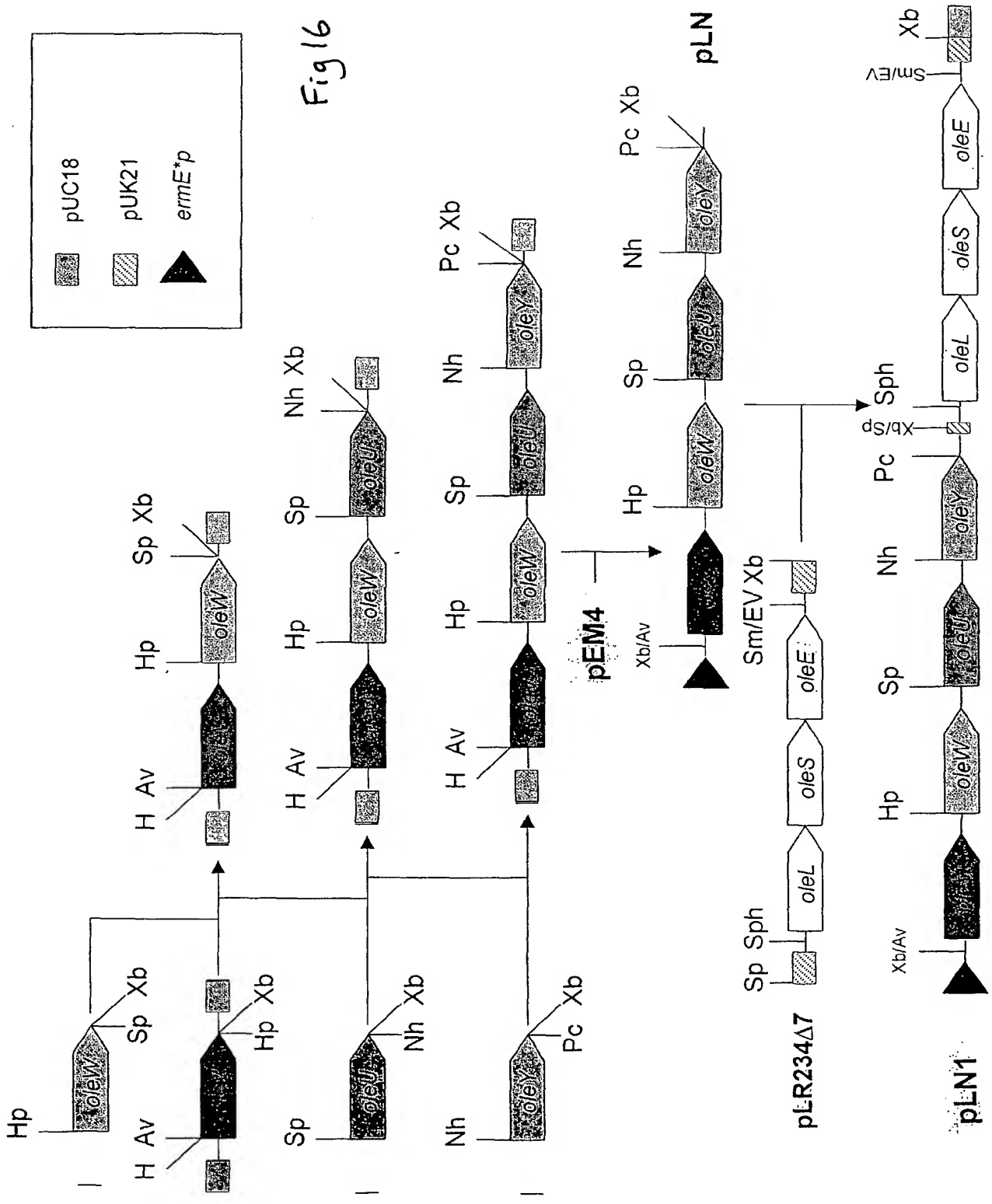

pUC18
pUK21
ermE*p
Hp
H Av
Sp Xb
Hp Xb
Nh Xb
Pc Xb
Sp Sph
Sm/EV Xb
Xb/Av
Xb/Sp
Sm/EV
Sph
oleW
oleU
oleY
oleL
oleS
oleE
pEM4
pLN
pLR234Δ7
pLN1

Fig 16

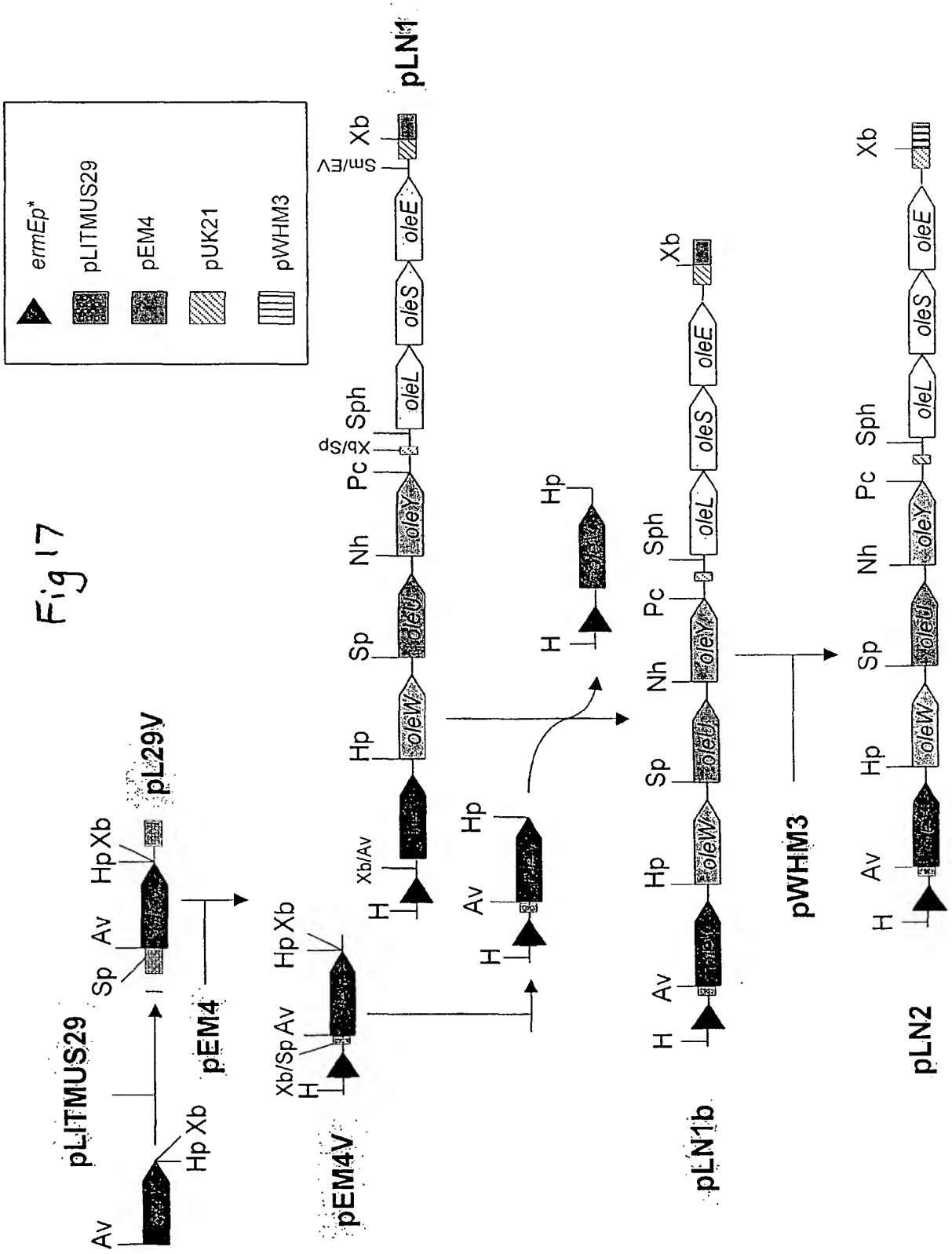

ermEp*
pLITMUS29
pEM4
pUK21
pWHM3
pLITMUS29
pL29V
pEM4
pEM4V
pLN1
pLN1b
pWHM3
pLN2
Av
Hp Xb
Sp
Xb/Sp
H
Xb/Av
Nh
Pc
Sph
Sm/EV
oleW
oleU
oleY
oleL
oleS
oleE

Fig 17

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/05426

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P19/62 C12N9/10 C07H17/08 C12N1/20 C12N15/54
C12N15/76 //(C12P19/62,C12R1:465)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | AGUIRREZABALAGA IGNACIO ET AL: "Identification and expression of genes involved in biosynthesis of L-oleandrose and its intermediate L-olivose in the oleandomycin producer Streptomyces antibioticus." ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, vol. 44, no. 5, May 2000 (2000-05), pages 1266-1275, XP002237961 ISSN: 0066-4804 cited in the application the whole document --- -/-- | 1-16 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

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'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

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'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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'&' document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 11 April 2003 | 29/04/2003 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Aslund, J |

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | MENDEZ C ET AL: "Altering the glycosylation pattern of bioactive compounds" TRENDS IN BIOTECHNOLOGY, ELSEVIER PUBLICATIONS, CAMBRIDGE, GB, vol. 19, no. 11, 1 November 2001 (2001-11-01), pages 449-456, XP004309138 ISSN: 0167-7799 cited in the application | |
| A | DOUMITH MICHEL ET AL: "Interspecies complementation in Saccharopolyspora erythraea: Elucidation of the function of oleP1, oleG1 and oleG2 from the oleandomycin biosynthetic gene cluster of Streptomyces antibioticus and generation of new erythromycin derivatives." MOLECULAR MICROBIOLOGY, vol. 34, no. 5, December 1999 (1999-12), pages 1039-1048, XP001021052 ISSN: 0950-382X cited in the application | |
| A | GAISSER S ET AL: "A DEFINED SYSTEM FOR HYBRID MACROLIDE BIOSYNTHESIS IN SACCHAROPOLYSPORA ERYTHRAEA" MOLECULAR MICROBIOLOGY, BLACKWELL SCIENTIFIC, OXFORD, GB, vol. 36, no. 2, 2000, pages 391-401, XP001021072 ISSN: 0950-382X | |
| A | WO 01 79520 A (LEADLAY PETER FRANCIS ;GAISSER SABINE (GB); STAUNTON JAMES (GB); B) 25 October 2001 (2001-10-25) the whole document | |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna[redacted]nal Application No
PCT/GB 02/05426

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 0179520 A | 25-10-2001 | AU 4858801 A | 30-10-2001 |
| | | EP 1278881 A1 | 29-01-2003 |
| | | WO 0179520 A1 | 25-10-2001 |